"Modified siRNAs for the study of the PAZ domain" Somoza, A., Terrazas, M., Eritja, R. Chem. Commun., 46(24), 4270-4272 (2010). doi: 10.1039/c003221b

Modified siRNAs for the study of the PAZ domain

Álvaro Somoza,*[,a] Montserrat Terrazas[b,c] and Ramon Eritja*[,b,c]

[a]IMDEA-Nanociencia, 28049, Madrid, Spain. E-mail: alvaro.somoza@imdea.org; Fax: +34 914976855; Tel: +34 914973654
[b]Institute for Advanced Chemistry of Catalonia, CSIC, C/Jordi Girona 18-26, E-08034, Barcelona, Spain
[c]Institut de Recerca Biomédica, CIBER-BBN, C/Baldiri Reixac 15, E-08028, Barcelona, Spain. E-mail: ramon.eritja@irbbarcelona.org; Fax: +34 932045904; Tel: +34 934039942

Abstract.
Chemical modifications aimed at stabilizing the interaction between the 3'-end of siRNAs and the PAZ domain of RISC have been tested for their effect on RNAi activity. Such modifications contribute positively to the stability of siRNAs in human serum.

RNA interference (RNAi) is a powerful gene regulatory process that allows the inhibition of genes in a very specific way.[1] This process is triggered by double stranded RNAs known as small interfering RNAs (siRNAs)[2] and is initiated with the recognition of siRNAs by the RNA-induced Silencing Complex (RISC), a protein complex located in the cytoplasm. First, RISC binds the siRNA duplex,[3] then, the passenger strand is cleaved and released to the cytoplasm.[4] On the other hand, the complementary strand, known as the guide strand, is used by the active complex to find the target sequence along the messenger RNA (mRNA) by base complementarity interaction. Finally, RISC induces endonucleotic cleavage of the target mRNA, preventing its translation into the corresponding protein.

During the last few years many efforts have been focused on the use of this technology as targeted therapy, since it could be useful for the treatment of diseases where the over-expression of genes is involved, such as cancer or inflammation related diseases.[5] In order to develop an effective treatment it would be desirable to achieve long lasting effects, good biodistribution and high tissue, organ or cell selectivity.[6] In this sense, several groups have modified siRNAs with a broad spectrum of chemical modifications in order to achieve long lasting effects and to prevent the degradation of siRNAs by RNases.[7]

In this paper we study the effect of novel modifications at the 3'-end of the guide strand of siRNAs with the aim of gathering structural information on the complex with RISC. In particular, the binding to a hydrophobic pocket of the PAZ domain of Argonaute protein (the key component of RISC) involved in the recognition of the 3'-end of the guide strand.

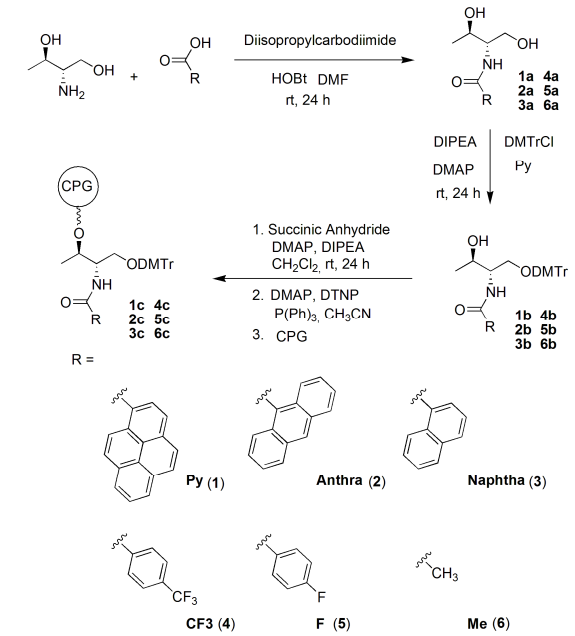
Crystallographic data reported for a fragment of the RISC complex have shown the presence of aromatic residues at the PAZ binding pocket.[8] Based on these data one could expect that the presence of aromatic derivatives at the 3'-end of the guide strand could stabilize its interaction with the PAZ pocket and therefore modulate the RNAi activity. Indeed, it could be considered that stabilizing the complex between the guide strand and the RISC complex would favour the gene inhibition process. Under this hypothesis Ueno and co-workers have explored the effect of aromatic modifications at the 3'-end of the guide strand on RNAi.[9] However, no significant differences were observed when siRNAs of the same length (21 nucleotides) were compared. On the other hand, crystal structures of the PAZ domain of Argonaute in complex with DNA–RNA duplexes reveal that the 30-end is released from the PAZ pocket during the cleavage of the mRNA.[10] The data suggest that this process is necessary to allow the protein complex to adopt the conformation required to perform RNase activity. Based on these results, one could expect negative effects on RNAi activity for stable PAZ:guide-strand complexes. In order to explore this hypothesis in more detail we have modified the 3'-end of the guide strand with aromatic derivatives of diverse size, shape and electronic properties, such as pyrene, anthracene, naphthalene, trifluoromethylbenzene and fluorobenzene.

Moreover, the stability of the modified siRNAs in serum has been evaluated.

The modified siRNAs at the 3'-end were prepared using functionalized Controlled Pore Glass (CPG) solid supports, which were employed in the automated RNA synthesis.

Derivatives **1a**–**5a** were prepared from L-threoninol and the corresponding carboxylic acids (Scheme 1). Additionally, acetic acid was utilized in the preparation of a non-aromatic derivative (**6a**), which was used as control of the aromatic interaction with the PAZ domain. The preparation of modified siRNAs started with the addition of a carboxylic acid to threoninol in the presence of the coupling reagents diisopropyl carbodiimide and *N*-hydroxybenzotriazole in DMF, which gave rise to the corresponding amide. The protection of the primary hydroxyl function with the dimethoxytrityl group was performed under standard conditions. Then, the solid supports were functionalized with threoninol derivatives. The addition of succinic anhydride and dimethylaminopyridine (DMAP) to the threoninol derivatives yielded the corresponding carboxylic acid. This group was activated by the addition of 2,2'-dithiobis-(5-nitropyridine), DMAP, and triphenylphosphine and utilized in the amide bond formation with the amino groups present in the CPG support.[11]

The modified solid supports were then employed in the preparation of RNA strands using a RNA synthesizer.[12] These strands were prepared to target the luciferase gene, which encodes for a bioluminescent protein commonly employed as reporter in gene inhibition assays.[2] (Guide strand: 5'-UCGAAGUAUUCCGCGUACGTT-3'; passenger strand: 5'-CGUACGCGGAAUACUUC GATT-3').



Scheme 1 Functionalization of solid support (CPG) with threoninol derivatives. HOBt: hydroxybenzotriazole; DMF: dimethylformamide; DMTrCl: 4,4'-dimethoxytrityl chloride; DIPEA: diisopropylethylamine; Py: pyridine; DMAP: 4-dimethylaminopyridine; DTNP: 2,2'-dithiobis- (5-nitropyridine); CPG: controlled pore glass.

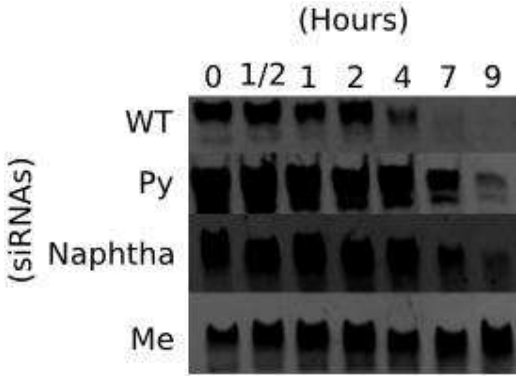


Fig. 1 Selected gels of siRNAs modified in the guide strand (see ESI for Anthra, CF3 and F gels). siRNAs were incubated with serum (50%) at 37 ºC. The samples were extracted with hot phenol and loaded in a denaturing gel.

RNA sequences were purified by HPLC and characterized by MALDI-TOF (see ESI). The siRNAs duplexes were prepared from a passenger and guide strand under proper annealing conditions.[2]

First, thermal stability of the modified siRNAs was compared with the wild-type siRNA (WT). Significant differences were not observed when the modification was in the guide strand (see ESI). On the other hand, when the modifications were located at the 3'-end of the passenger strand a small increase in stability was observed compared with the WT.

Then, the stability of the modified siRNAs in serum was evaluated. For this purpose, siRNAs were incubated in serum (50%) at 37 ºC for different periods of time. The samples were extracted with hot phenol and loaded into a polyacrylamide gel for analysis. The WT siRNA showed a significant decrease in stability after 4 and 7 h and it was completely

degraded by nucleases after 9 h in serum. The stability of modified siRNAs was higher than that of WT siRNA (Fig. 1). Among them, double stranded siRNA having aMe group at the 30-end of the guide strand displayed strongly enhanced stability, with significant siRNA population after 24 h in serum. This is a notable result for just a single modification in RNA (see ESI for complete gels).

The activity of modified siRNAs was evaluated using the dual-luciferase assay system at different concentrations and for different periods of time. Only when very low concentrations of siRNAs were employed could some differences be observed: 320 pM and 32 pM in SH-SY5Y and in HeLa cells respectively. At these concentrations, siRNAs with modified guide strand showed significant differences in activity compared to WT (Fig. 2a and c). The pyrene, anthracene and naphthalene derivatives showed a decrease in activity. Meanwhile the other derivatives (CF3, F and Me) showed an activity close to the siRNA without modifications (WT). The results herein observed are likely to be due to the interaction of the modified guide strand with the binding pocket at the PAZ domain, since the experiments with modified passenger strand showed WT activity in all cases (Fig. 2b and d). In addition, the decrease in RNAi activity observed cannot be related to the degradation of the siRNAs due to the presence of RNases, because modified siRNAs exhibited better stability in serum than WT.

The results could be explained based on stabilization effects due to the aromatic modifications. The pyrene, anthracene and naphthalene derivatives could favour π-stacking interaction with the aromatic residues of the binding pocket, such as Phe292, Tyr309 and Tyr277. Therefore, based on the report by Patel where the 3'-end should be released from the PAZ pocket during the RNAi process,[10] an increase in stabilization could cause a decrease in RNAi activity.

An alternative explanation could involve destabilizing steric effects. The modifications employed could be too big for the pocket thus promoting the dissociation of the complex between the protein and the RNA strand, which would lead to a decrease in activity. However, the crystal structure of the PAZ domain of Argonaute 2 in complex with a 9-mer siRNA-like duplex reported by Patel and co-workers (PDB:1si3)[8a] reveals that these modifications should fit in that pocket and therefore the steric effects should not be critical in this case. In addition, we performed docking experiments using autodock-vina[13] on the PAZ domain with the aromatic moieties. The predicted binding affinity found for the aromatic rings showed a trend that matches with the observed activity: -7.9, -7.7, -6.5, -6.4 and-5.8 kcal/mol for pyrene, anthracene, naphthalene, trifluoromethyl benzene and fluorobenzene, respectively. These results suggest that the pyrene derivative should bind tighter than the other derivatives to the PAZ domain, and therefore present the lowest activity.

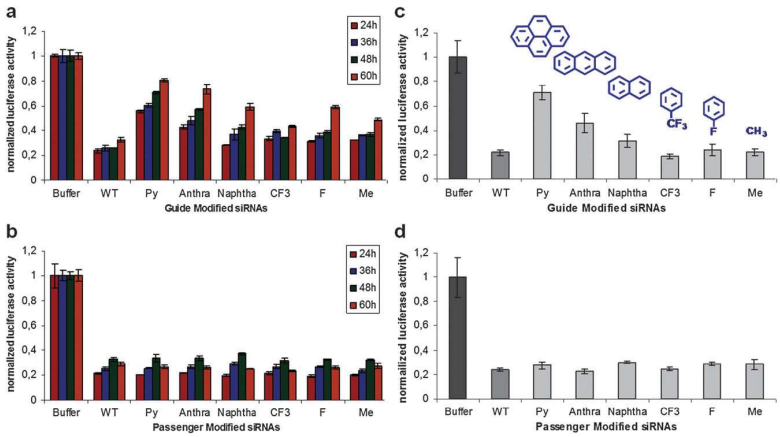


Fig. 2 RNA interference activity of modified siRNAs. (a) Activity of guide modified siRNAs at 320 pM in SH-SY5Y cells. (b) Activity of passenger modified siRNAs at 320 pM in SH-SY5Y. (c) Activity of guide modified siRNAs at 32 pM in HeLa cells after 24 h. (d) Activity of passenger modified siRNAs at 32 pM in HeLa cells after 24 h.

In view of these results, we propose that the difference in activity observed in these examples is more likely to be due to the stabilization through interaction with aromatic residues of the pocket than to destabilization due to steric effects.

In summary, we have studied the effect of aromatic derivatives of different size at the 3'-end of siRNAs on RNAi activity. It has been found that RNAi activity is modulated by the substituents located at the 3'-end of the guide strand. Thus, this report provides a complementary strategy to gather information on the binding pocket of the PAZ domain of RISC, which could be used to design more efficient siRNAs. Moreover, we have studied the effect of these modifications on the serum stability of the corresponding modified siRNAs. We have found that a single modification at the 3'-end of the guide strand can significantly stabilize the corresponding siRNA in serum. Among the modifications tested, the methyl modification causes a significant increase in serum stability. Moreover, this modification does not adversely affect gene silencing activity, which makes it an ideal modification for applications in targeted therapy by RNA interference.

Notes and references

1 A. Fire, S. Xu, M. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello, Nature, 1998, 391, 806.
2 S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl, Nature, 2001, 411, 494.
3 M. Jinek and J. A. Doudna, Nature, 2009, 457, 405.
4 C. Matranga, Y. Tomari, C. Shin, D. P. Bartel and P. D. Zamore, Cell, 2005, 123, 607.
5 D. Castanotto and J. J. Rossi, Nature, 2009, 457, 426.
6 J. Kurreck, Angew. Chem., Int. Ed., 2009, 48, 1378.
7 (a) M. Manoharan, Curr. Opin. Chem. Biol., 2004, 8, 570–579; (b) M. Dutkiewicz, H. P. Grunert, H. Zeichhardt, S. W. Lena, J. Wengel and J. Kurreck, FEBS Lett., 2008, 582, 3061; (c) M. Fisher, M. Abramov, A. V.

Aerschot, D. Xu, R. L. Juliano and P. Herdewijn, Nucleic Acids Res., 2007, 35, 1064; (d)M. Terrazas and E. T. Kool, Nucleic Acids Res., 2008, 37, 346; (e) J. B. Bramsen,M. B. Laursen, A. F. Nielsen, T. B. Hansen, C. Bus, N. Langkjaer, B. R. Babu, T. Højland, M. Abramov, A. Van Aerschot, D. Odadzic, R. Smicius, J. Haas, C. Andree, J. Barman, M. Wenska, P. Srivastava, C. Zhou, D. Honcharenko, S. Hess, E. Mu¨ ller, G. V. Bobkov, S. N. Mikhilov, E. Fava, T. F. Meyer, J. Chattopadhyaya, M. Zerial, J. W. Engels, P. Herdewijn, J. Wengel and J. Kjems, Nucleic Acids Res., 2009, 37, 2867.
8 (a) J.-B. Ma, K. Ye and D. J. Patel, Nature, 2004, 429, 318; (b) J.-J. Song, S. K. Smith, G. J. Hannon and L. Joshua-Tor, Science, 2004, 305, 1434.
9 Y. Ueno, Y. Watanabe, A. Shibata, K. Yoshikawa, T. Takano, M. Kohara and Y. Kitade, Bioorg. Med. Chem., 2009, 17
1974.
10 Y. Wang, S. Juranek, H. Li, G. Sheng, G. S. Wardle, T. Tuschl and D. J. Patel, Nature, 2009, 461, 754.
11 K. C. Gupta, P. Kumar, D. Bhatia and A. K. Sharma, Nucleosides, Nucleotides Nucleic Acids, 1995, 14, 829.
12 A. Somoza, Chem. Soc. Rev., 2008, 37, 2668.
13 (a) O. Trott and A. J. Olson, J. Comput. Chem., 2009, 31, 455; (b) M. F. Sanner., J. Mol. Graphics Mod., 1999, 17, 57.

ELECTRONIC SUPPLEMENTARY INFORMATION (ESI).

CONTENTS:

General Procedures:

General experimental methods: Reagents were purchased from Aldrich and used without further purification. Thin layer chromatography was carried out using Silica Gel 60 F254 plates. Column chromatography was performed using Silica Gel (60 Å, 230 x 400 mesh). All NMR spectra were recorded on Varian Mercury 400 MHz (Unitat d'RMN, Serveis Cientificotècnics, Parc Cientific de Barcelona) instrument as solutions in the deuterated solvent indicated, and the chemical shifts are reported in parts per million (ppm). Coupling constants are reported in hertz (Hz). HRMS were performed on a LC/MSD-TOF (Agilent technologies) high resolution mass spectrometer by Servei d'Espectrometria de Masses (Universitat de Barcelona).

Procedure A: Amide Formation :
To a solution of the corresponding acid (1.2 equiv) in DMF (0.5 M) at room temperature, *N*-hydroxybenzotriazole (1.1 equiv) and diisopropylcarbodiimide (1.1 equiv) were added. After stirring the mixture for 5 minutes D- or L-Threoninol (1 equiv) was added. The resulting mixture was stirred at room temperature for 24 h and then quenched by the addition of methanol. The solvent was evaporated under vacuum and the residue purified by flash chromatography.

Procedure B: DMTr Protection:
To a solution of the corresponding diol (1 equiv) in pyridine (0.2 M) at 0º C, diidopropylethylamine (1.5 equiv), 4,4´-dimethoxyltritylchloride (1.2 equiv) and dimethylaminopyridine (catalytic amount) were added. After 15 min the mixture was allowed to reach room temperature, then it was stirred for 24 h and finally the reaction was quenched with methanol. The solvent was evaporated under vacuum and the residue purified by flash chromatography.

Procedure C: CPG Functionalization (Two Steps) (Gupta, K. C.; Kumar, P.; Bhatia, D.; Sharma, A. K. Nucleosides and Nucleotides, **1995**, *14*, 829-832):

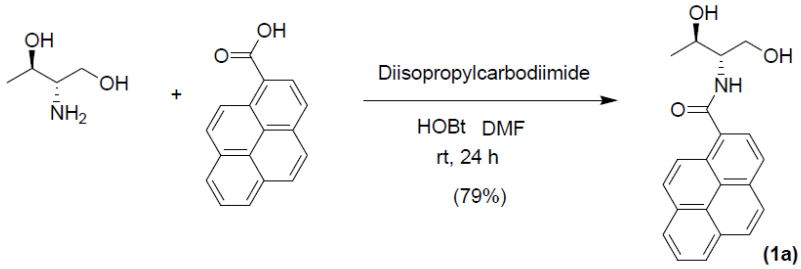
I To a solution of the corresponding alcohol (1 equiv) in dichloromethane (0.2 M) succinic anhydride (1.3 equiv), diisopropylethylamine (1.4 equiv) and dimethyaminopyridine (catalytic amout) were added. The solution was stirred for 24 h at room temperature. Then, the solution was washed with a solution of sodium dihydrogen phosphate (1%). The organic layer was dried over Magnesium sulfate, filtered and evaporated to yield a yellowish oil.

II To a solution of DMAP (1.5 equiv) in acetonitrile (60 mM) the product obtained in step II was added (1 equiv). After mixing it well (vortex), the solution was added to a solution of 2,2´-dithiobis-(5-nitropyridine) (1.5 equiv). The solution was mixed well and added to triphenyl phosphine (1.5 equiv). The mixture was vortexed till all reagents were dissolved giving rise to a reddish solution, which was added to 200 mg of CPG (500 Á). After 1 h at room temperature the solution was removed and the CPG washed with methanol (3 X 20 mL) and, dry acetonitrile (3 x 20 mL). Once the CPG was dry, 2 mL of a 1:1 mixture of the capping reagents utilized on oligonucleotide synthesis (Cap Mix A: Acetic anhydride/Py/THF; Cap Mix B: 1-Methylimidazole /THF; from GlenResearch) was added. After 15 min at room temperature, the CPG was washed with dry

acetontrile (6 x 20 mL) and dried. The CPG loading was calculated by detrytylation of a sample as follows: 10 mg of CPG were treated with 5 mL of a detrytilation solution (3mL of perchloric acid and 2 mL of ethanol) for 30 min. Then 200 µL of the mixture were dissolved in 800 µL of the detrytilation solution and absorbance was measured at 498. Functionalization (F) was determined by Lambert-Beer law. F = (ABS x V) / (ε x gr) = M/ gr.

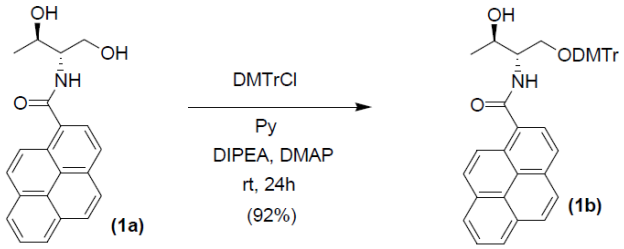
CHARACTERIZATION OF ORGANIC COMPOUNDS

N-[(2*S*, 3*R*)-1,3-dihydroxybutan-2-yl]pyrene-1-carboxamide (1a).



Compound **1a** was obtained following procedure A. After flash chromatography (cyclohexane 4 / CH_2Cl_2 4 / MeOH 0.5) 302 mg (0.906 mMol) were obtained as a white solid (79%). ^{1}H NMR (400 MHz, CD_3OD): 8.53 (d, *J* = 9.3 Hz, 1H), 8.25 (t, *J* = 7.5 Hz, 3H), 8.21-8.02 (m, 5H), 4.28 (dt, *J* = 6.2 and 3.6 Hz, 1H), 4.18 (dq, *J* = 6.4 and 3.6 Hz, 1H), 3.87 (System ABX, J_{AB}= 11.1 Hz, J_{AX} = 5.8 Hz, and J_{Bx}= 6.6 Hz, 2H), 1.38 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): 173.4, 133.9, 132.8, 132.6, 132.1, 129.7, 129.6, 129.5, 128.3, 127.6, 126.9, 126.8, 126.1, 125.8, 125.61, 125.59, 125.51, 67.5, 63.0, 58.5, 20.8. HRMS m/z: Calc for $C_{21}H_{20}NO_3$ (M + H^+) 334.1437, found 334.1449.

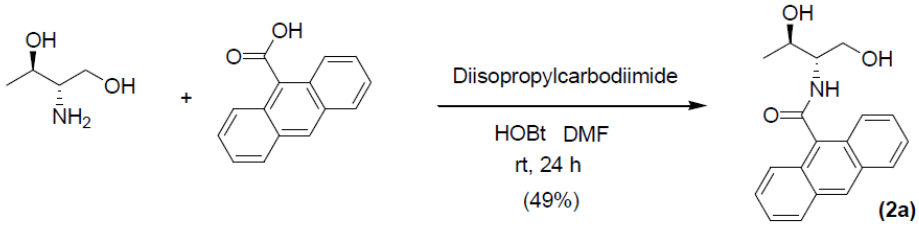
N-[(2*S*, 3*R*)-1-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-hydroxybutan-2-yl)-pyrene-1-carboxamide (1b).



Compound **1b** was obtained following procedure B. After flash chromatography (cyclohexane 2 / EtOAc 1) 133 mg (0.209 mMol) were obtained as a white solid and 24 mg of the starting material were recovered. (70% yield, or 92% yield based on the recovered starting material). ^{1}H NMR (400 Mz, $CDCl_3$): 8.62 (d, *J* = 9.3 Hz, 1H), 8.21 (d, *J* = 7.7 Hz, 2H), 8.12 (s, 3H), 8.09 (d, *J* = 2.1 Hz, 1H), 8.04 (t, *J* = 7.7 Hz, 2H), 7.45 (d, *J* = 7.6 Hz, 2H), 7.34 (d, *J* = 8.7 Hz, 4H), 7.28 (t, *J* = 7.7 Hz, 2H), 7.20 (t, *J* = 7.2 Hz, 1H), 6.89 (d, *J* = 8.7 Hz, 1H), 6.81 (dd, *J* = 8.9 and 2.8 Hz, 4H), 4.38-4.32 (m, 1H), 4.28-4.22 (m, 1H), 3.73 (s, 3H), 3.72

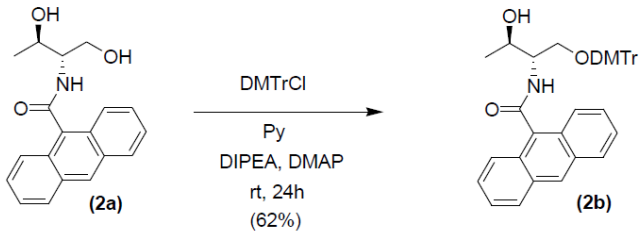
(s, 3H), 3.63 (ABX System, J_{AB} = 9.6 Hz, J_{AX} = 3.7 Hz, J_{BX} = 3.6 Hz, 2H), 1.35 (d, *J* = 6.3 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 170.2, 158.6, 158.5, 144.3, 135.4, 135.2, 132.6, 131.1, 130.7, 130.6, 129.9 (4C), 128.7, 128.6, 128.5, 128.0 (2C), 127.9 (2C), 127.0, 126.9, 126.3, 125.8, 125.7, 124.7, 124.5, 124.5, 124.3, 124.2, 113.3 (4C), 86.9, 69.0, 65.6, 55.1 (2C), 54.3, 20.3. HRMS m/z: Calc for $C_{42}H_{37}NO_5Na$ ($M + Na^+$) 658.2563, found 658.2563.

***N*-[(2*S*, 3*R*)-1,3-dihydroxybutan-2-yl)anthracene-9-carboxamide (2a).**



Compound **2a** was obtained following procedure A. After flash chromatography (cyclohexane 6 / CH_2Cl_2 3 / MeOH 0.5) 273 mg (1.052 mMol) were obtained as a yellowish solid (49%). ^{1}H NMR (400 MHz, CD_3OD): 8.56 (s, 1H), 8.27-8.15 (m, 2H), 8.06 (d, *J* = 8.3 Hz, 2H), 7.57-7.47 (m, 4H), 4.40 (ddd, *J* = 6.7, 5.6 and 3.9 Hz, 1H), 4.15 (dq, *J* = 6.4 and 3.8 Hz, 1H), 3.87 (ABX System: J_{AB} = 11.0 Hz, J_{AX} = 5.6 Hz, J_{BX} = 6.9 Hz, 2H), 3.35 (s, 1H), 1.39 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, D_3OD): 172.7, 133.5, 132.7, 129.6 (2C), 129.3, 129.2 (2C), 127.7 (2C), 126.6 (4C), 126.4, 67.4, 63.0, 58.8, 20.9. HRMS m/z: Calc for $C_{19}H_{19}NNaO_3$ $(M + Na)_+$ 332.1257, found 332.1258.

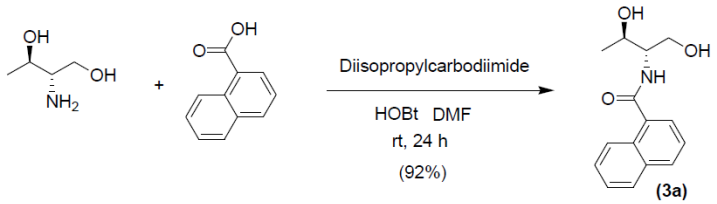
***N*-[(2*S*, 3*R*)-1-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-hydroxybutan-2-yl]-anthracene-9-carboxamide (2b).**



Compound **2b** was obtained following procedure B. After flash chromatography (cyclohexane 3 / EtOAc 1) 130 mg (0.213 mMol) were obtained as a white solid (62%). ^{1}H NMR (400 Mz, $CDCl_3$): 8.48 (s, 1H), 8.11 (bs, 1H), 8.01 (d, J = 9.2 Hz, 2H), 7.47 (t, J = 8.2 Hz, 2H), 7.41 (d, J = 7.2 Hz, 2H), 7.31 (d, J = 8.8 Hz, 4H), 7.26 (t, J = 7.4 Hz, 2H), 7.21 (d, J= 7.0 Hz, 1H), 6.80 (d, J = 8.8 Hz, 4H), 6.73 (d, J = 8.8 Hz, 1H), 4.49 (td, J = 7.3 and 3.9 Hz, 1H), 4.16 (m, 1H), 3.77 (s, 3H), 3.76 (s, 3H), 3.62 (ABX System, JAB = 9.6 Hz, JAX = 3.6 Hz, JBX = 4.4 Hz, 2H), 3.07 (s, 1H), 1.35 (d, J = 6.3 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 169.9, 158.6 (2C), 144.2 (2C), 135.4, 135.1, 131.7, 131.1 (2C), 130.0 (3C), 129.9 (3C), 128.5 (2C), 128.3, 128.1, 128.0 (2C), 127.9 (2C), 127.0, 126.7 (2C),

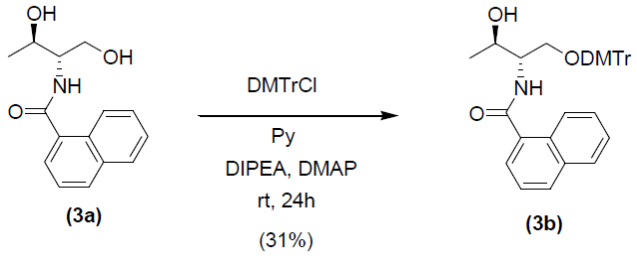
125.4, 125.2, 113.3 (2C), 113.2 (2C), 86.9, 68.8, 65.5, 55.2 (2C), 54.6, 20.6. HRMS m/z: Calc for C40H37NNaO5 (M + Na)+ 634.2564, found 634.2570.

N-[(2S, 3R)-1,3-dihydroxybutan-2-yl]-1-naphthamide (3a).



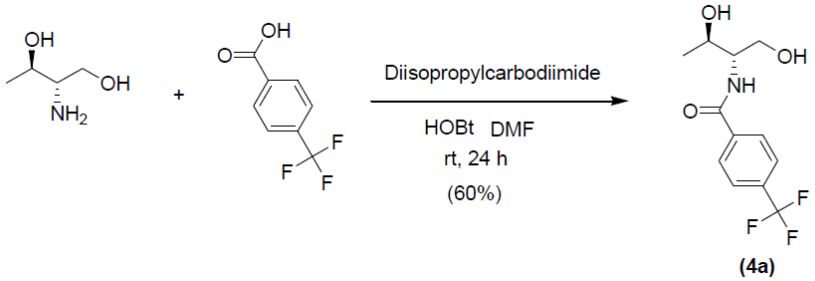
Compound **3a** was obtained following procedure A. After flash chromatography (cyclohexane 6 / CH_2Cl_2 3 / MeOH 0.5) 273 mg (1.052 mMol) were obtained as a yellowish solid (92%). ^{1}H NMR (400 MHz, CD_3OD): 8.26 (d, *J* = 9.1 Hz, 1H), 7.95 (d, *J* = 8.3 Hz, 1H), 7.90 (d, *J* = 7.3 Hz, 1H), 7.69 (d, *J* = 7.0 Hz, 1H), 7.57-7.47 (m, 3H), 4.21-4.16 (m, 1H), 4.15-4.09 (m, 1H), 3.79 (System ABX; J_{AB} = 11.1 Hz, J_{AX} = 5.8 Hz, J_{BX} = 6.5 Hz, 2H), 1.30 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): 173.1, 136.1, 135.3, 131.7, 131.6, 129.6, 128.2, 127.6, 126.6, 126.5, 126.1, 67.6, 63.1, 58.4, 20.9. HRMS m/z: Calc for $C_{15}H_{18}NO_3$ (M + H_+) 260.1281, found 260.1291.

***N*-[(2S, 3*R*)-1-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-hydroxybutan-2-yl]- 1- naphthamide (3b).**



Compound **3b** was obtained following procedure B. After flash chromatography (cyclohexane 3 / EtOAc 1) 146 mg (0.259 mMol) were obtained as a white solid (31%). ^{1}H NMR (400 Mz, $CDCl_3$): 8.37-8.32 (m, 1H), 7.95 (d, *J* = 8.2 Hz, 1H), 7.92- 7.87 (m, 1H), 7.66 (dd, *J* = 7.0 and 1.1 Hz, 1H), 7.56-7.52 (m, 2H), 7.48 (dd, *J* = 8.2 and 7.1 Hz, 1H), 7.40 (d, *J* = 7.2 Hz, 2H), 7.31 (d, *J* = 8.9 Hz, 4H), 7.26 (t, *J* = 7.3 Hz, 2H), 7.20 (t, *J* = 7.1 Hz, 1H), 6.80 (dd, *J* = 8.8 and 3.7 Hz, 4H), 6.71 (d, *J* = 8.8 Hz, 1H), 4.28-4.24 (m, 1H), 4.23-4.18 (m, 1H), 3.76 (s, 3H), 3.75 (s, 3H), 3.56 (System ABX, J_{AB} = 9.7 Hz, J_{AX} = 3.8 Hz, J_{BX} = 3.6 Hz, 2H), 3.17 (s, 1H), 1.29 (d, *J* = 6.34 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 169.8, 158.7, 158.6, 144.2, 135.4, 135.2, 134.4, 133.7, 130.7, 130.1, 129.9 (4C), 128.3, 128.0 (2C), 127.9 (2C), 127.2, 127.0, 126.4, 125.5, 124. 9, 124.7, 113,3 (4C), 86.9, 69.1, 65.7, 55.2 (2C), 53.9, 20.2. HRMS m/z: Calc for $C_{36}H_{35}NNaO_5$ (M + Na)+ 584.2407, found 584.2402.

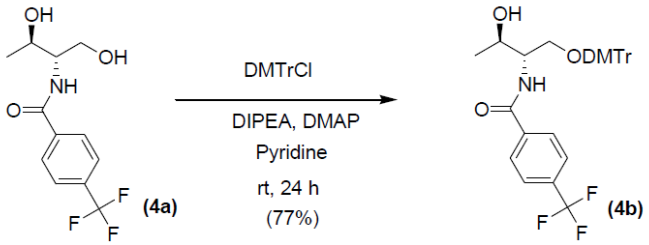
***N*-[(2*S*, 3*R*)-1,3-dihydroxybutan-2-yl)-4-(trifluoromethyl]benzamide (4a).**



(4a)

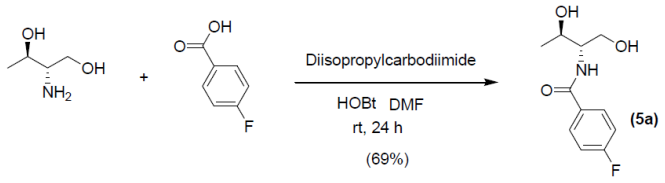
Compound **4a** was obtained following procedure A. After flash chromatography (cyclohexane 4 / CH_2Cl_2 4/ MeOH 0.5) 157 mg (0.566mMol) were obtained as a white solid (60%). ^{1}H NMR (400 MHz, CD_3OD): 8.03 (d, *J* = 8.2 Hz, 2H), 7.78 (d, *J* = 8.2 Hz, 2H), 4.14-4.04 (m, 2H), 3.75 (System ABX: J_{AB} = 11.2 Hz, J_{AX} = 5.6 Hz, J_{BX} = 6.2 Hz, 2H), 1.22 (d, *J* = 6.2 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): 169.4, 139.7, 134.1 (q, J_{F-C} = 32.4 Hz, 1C), 129.3 (2C), 126.5 (q, J_{F-C} = 3.8 Hz, 2C), 124.0, 67.3, 62.8, 58.4, 20.6. ^{19}F NMR (376 MHz, CD_3OD): -64.86. HRMS m/z: Calc for $C_{12}H_{15}NO_5F_3$ $(M + H)^+$ 278.0998, found 278.0999.

***N*-[(2*S*, 3*R*)-1-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-hydroxybutan-2-yl]-4- (trifluoromethyl)benzamide (4b).**



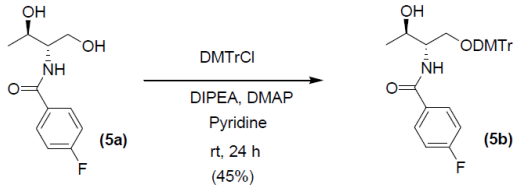
Compound **4b** was obtained following procedure B. After flash chromatography (cyclohexane 3 / EtOAc 1) 580 mg (0.828 mMol) were obtained as a white solid (77%). ^{1}H NMR (400 Mz, $CDCl_3$): 7.89 (d, *J* = 8.1 Hz, 2H), 7.73 (d, *J* = 8.2 Hz, 2H), 7.38 (d, *J* = 7.2 Hz, 2H), 7.30-7.18 (m, 4H), 6.79 (dd, *J* = 8.8 and 6.8 Hz, 2H), 4.28- 4.20 (m, 1H), 4.15-4.10 (m, 1H), 3.76 (s, 3H), 3.75 (s, 3H), 3.49 (System ABX: J_{AB} = 9.8 Hz, J_{AX} = 4.2 Hz, J_{BX} = 3.4 Hz, 2H), 3.07 (s, 1H), 1.21 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 166.3, 158.6 (2C), 144.2, 137.6, 135.3, 135.2, 133.3 (q, J_{FC} = 32.8 Hz, 1C), 129.9 (2C), 129.8 (2C), 128.0 (2C), 127.8 (2C), 127.4 (2C), 127.0, 125.7 (q, J_{F-C} = 3.8 Hz, 2C), 124.9, 113.3 (2C), 113.2 (2C), 86.9, 68.5, 65.2, 55.1(2C), 54.0, 20.1. ^{19}F NMR (376 MHz, $CDCl_3$): -63.36. HRMS m/z: Calc for $C_{33}H_{32}F_3NNaO_5$ $(M + Na)^+$ 602.2125, found 602.2126.

***N*-[(2*S*, 3*R*)-1,3-dihydroxybutan-2-yl]-4-fluorobenzamide (5a).**



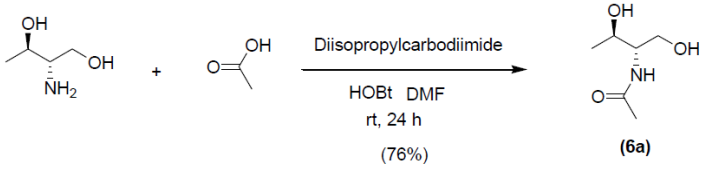
Compound **5a** was obtained following procedure A. After flash chromatography (cyclohexane 5 / CH_2Cl_2 4 / MeOH 0.5) 180 mg (0.792 mMol) were obtained as a white solid (69%). ^{1}H NMR (400 MHz, CD_3OD): 7.92 (dd, *J* = 8.8 and 5.4 Hz, 2H), 7.20 (t, *J* = 8.7 Hz, 2H), 4.13-4.02 (m, 2H), 3.73 (System ABX, J_{AB} = 11.2 Hz, J_{AX} = 5.9 Hz, J_{BX} = 6.3 Hz, 2H), 1.21 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): 169.5, 166.2 (d, J_{F-C} = 250.5 Hz, 1C) , 132.1, 131.0 (d, J_{F-C} = 8.9 Hz, 2C), 116.4 (d, J_{F-C} = 22.1Hz, 2C), 67.3, 62.8, 58.2, 20.6. ^{19}F NMR (376 MHz, CD_3OD): -111.2. HRMS m/z: Calc for $C_{11}H_{15}NO_3F$ (M + H^+) 228.1030, found 228.1035.

_N_-[(2_S_, 3_R_)-1-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-hydroxybutan-2-yl]-4-fluorobenzamide (5b).



Compound **5b** was obtained following procedure B. After flash chromatography (cyclohexane 3 / EtOAc 1) 313 mg (0.591 mMol) were obtained as a white solid (45%). ^{1}H NMR (400 Mz, $CDCl_3$): 7.81 (dd, *J* = 8.8 and 5.3 Hz, 2H), 7.37 (d, *J* = 7.1 Hz, 2H), 7.30-7.18 (m, 7H), 7.14 (t, *J* = 8.6 Hz, 2H), 6.79 (dd, *J* = 8.9 and 6.8 Hz, 4H), 4.21 (d, *J* = 6.3 Hz, 1H), 4.15-4.05 (m, 1H), 3.77 (s, 3H), 3.76 (s, 3H), 3.56 (dd, *J* = 9.7 and 4.2 Hz, 1H), 3.38 (dd, *J* = 9.7 and 3.4 Hz, 1H), 3.13 (s, 1H), 1.20 (d, *J* = 6.37 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 166.6, 164 (d, J_{F-C} = 252 Hz, 1C), 158.6 (2C), 144.2, 135.4, 135.2, 130.5 (d, J_{F-C} = 3.2 Hz, 1C), 129.9 (2C), 129.8 (2C), 129.3 (d, J_{F-C} = 8.9 Hz, 2C), 128.0 (2C), 127.8 (2C), 127.0, 115.6 (d, J_{F-C} = 21.8 Hz, 2C), 113.3 (4C), 86.9, 68.9, 65.5, 55.2 (2C), 53.9, 20.0. ^{19}F NMR (376 MHz, $CDCl_3$): -108.44. HRMS m/z: Calc for $C_{32}H_{32}NO_5FNa$ (M + Na^+) 552.2156, found 552.2143.

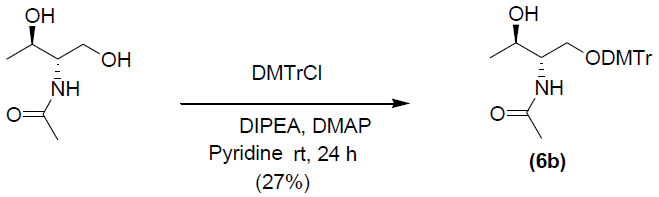
_N_-[(2_S_, 3_R_)-1,3-dihydroxybutan-2-yl]-acetamide (6a).



Compound **6a** was obtained following procedure A. After flash chromatography (cyclohexane 4 / CH_2Cl_2 4 / MeOH 0.5) 159 mg (1.08 mMol) were obtained as a

colorless oil (76%). $_1$H NMR (400 MHz, CD_3OD): 4.04-3.94 (m, 1H), 3.84-3.76 (m, 1H), 3.61 (System ABX, J_{AB} = 11.0 Hz, J_{AX} = 5.9 Hz, J_{BX} = 6.3 Hz, 2H), 2.00 (s, 3H), 1.14 (d, J = 6.4 Hz, 3H). $_{13}$C NMR (100 MHz, CD_3OD): 173.8, 67.2, 62.8, 57.6, 22.7, 20.4. HRMS m/z: Calc for $C_6H_{11}NO_2$ (M – H_2O) 129.0790, found 129.1524.

***N*-[(2*S*, 3*R*)-1-(bis(4-methoxyphenyl)(phenyl)methoxy)-3-hydroxybutan-2-yl]-acetamide (6b).**



Compound **6b** was obtained following procedure B. After flash chromatography (cyclohexane 1 / EtOAc 3) 85 mg (0.189 mMol) were obtained as a yellowish solid. $_1$H NMR (400 Mz, $CDCl_3$): 7.38 (d, J = 7.3 Hz, 2H), 7.27 (d, J = 8.8 Hz, 6H), 7.22 (t, J = 7.2 Hz, 1H), 6.83 (d, J = 8.74 Hz, 4H), 6.05 (d, J = 8.7 Hz, 1H), 4.15-4.06 (m, 1H), 3.94-3.88 (m, 1H), 3.79 (s, 6H), 3.35 (System ABX, J_{AB} = 9.7 Hz, J_{AX} = 4.4 Hz, J_{BX} = 3.5 Hz, 2H), 3.02 (s, 1H), 2.02 (s, 3H), 1.13 (d, J = 6.4 Hz, 3H). $_{13}$C NMR (100 MHz, $CDCl_3$): 170.4, 158.7 (2C), 144.3, 135.5, 135.3, 129.9 (2C), 129.8 (2C), 128.0 (2C), 127.9 (2C), 127.0, 113.3 (4C), 86.9, 68.7, 65.4, 55.2 (2C), 53.4, 23.3, 19.9. HRMS m/z: Calc for $C_{27}H_{31}NNaO_5$ $(M + Na)^+$ 472.2100, found: 472.2096.

RNA synthesis and purification methods:

RNA oligonucleotides were synthesized in a ABI 394 DNA / RNA Synthesizer using 2'-TBDMS phosphoramidites (Sigma-Aldrich). RNA oligonucleotides were synthesized on the 1 µmol scale. After solid-phase synthesis, the solid support was transferred to a screw-cap glass vial and incubated at 55 ºC for 1 h with 1.5 mL of NH_3 solution (33%) and 0.5 mL of ethanol. After the vial was cooled briefly on ice, the supernatant was transferred by pipet into 2 mL Eppendorf tubes; the solid support and vial were rinsed with 50% ethanol (2 × 0.25 mL). The combined solutions were evaporated to dryness using an evaporating centrifuge. The residue was dissolved in a total volume of 0.33 mL of TBAF (1M) in THF and rocked at room temperature for 12 h. Then, 0.33 mL of Et_3NHAcO (1 M) and 0.33 mL of water were added to the solution, and the oligonucleotide was desalted on a NAP-10 column using water as the eluent and evaporated to dryness. The oligonucleotides were purified by HPLC (DMT-ON). The pure fractions were combined and evaporated to dryness. The residues were treated with 0.4 mL of AcOH solution (3%) and incubated at room temperature for 1 h. The deprotected oligonucleotides were purified by HPLC (DMT-OFF). The pure fractions were combined and evaporated to reach a volume of 0.5 mL and desalted using a NAP-5 column and water as eluent.

Oligonucleotide Charaterization.

All synthesized oligonucleotides were characterized by MALDI-TOF mass spectrometry:

RNA	Sequence (5'-3')	MW calculated	MW found
WT GS	UCGAAGUAUUCCGCGUACGTT	6646.2	6646.2
Py GS	UCGAAGUAUUCCGCGUACGT-**Py**	6736.1	6731.1
Anthra GS	UCGAAGUAUUCCGCGUACGT-**Anthra**	6712.1	6707.8
Naph GS	UCGAAGUAUUCCGCGUACGT-**Naph**	6662.1	6657.4
CF3 GS	UCGAAGUAUUCCGCGUACGT-**CF3**	6680.1	6673.3
F GS	UCGAAGUAUUCCGCGUACGT-**F**	6630.1	6625.6
Me GS	UCGAAGUAUUCCGCGUACGT-**Me**	6550.1	6550.9
WT PS	CGUACGCGGAAUACUUCGATT	6669.2	6669.4
Py PS	CGUACGCGGAAUACUUCGAT-**Py**	6759.1	6753.5
Anthra PS	CGUACGCGGAAUACUUCGAT-**Anthra**	6735.1	6735.3
Naph PS	CGUACGCGGAAUACUUCGAT-**Naph**	6685.1	6686.4
CF3 PS	CGUACGCGGAAUACUUCGAT-**CF3**	6703.1	6707.4
F PS	CGUACGCGGAAUACUUCGAT-**F**	6653.1	6656.6
Me PS	CGUACGCGGAAUACUUCGAT-**Me**	6573.1	6576.9

GS: Guide Strand; PS: Passenger Strand.

Thermal Stability of siRNAs

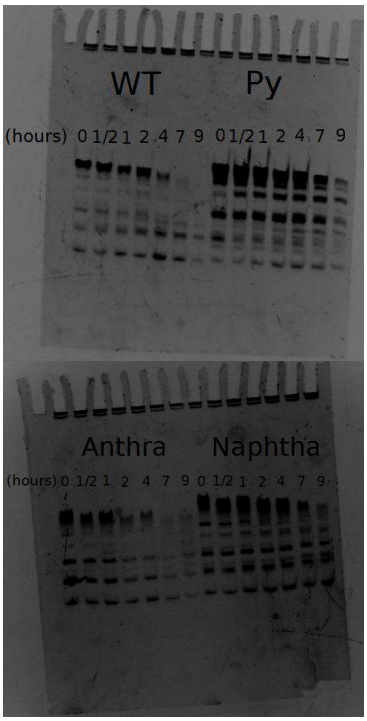
For annealing of siRNAs, a solution of guide and passenger strand at 20µM in annealing buffer (100mM potassium acetate, 30mM HEPES-KOH at pH 7.4, 2mM magnesium acetate) were incubated for 1 min at 92 ºC and cooled down slowly to room temperature.

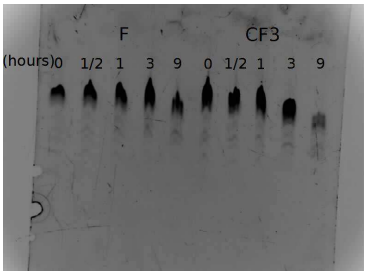
SiRNAs (0.5 µM each) were dissolved in buffer (50 mM potassium acetate, 1 mM magnesium acetate, 15 mM HEPES-KOH at pH 7.4). Experiments were performed in Teflon-stoppered 1 cm path length quartz cells on a JASCO V-650 spectrophotometer equipped with thermoprogrammer. The denaturation experiments were done at a rate of 1 °C/min to 90 ℃, monitoring absorbance at 260 nm. In all cases the complexes displayed sharp, apparently two-state transitions. The data were analyzed by the denaturation curve processing program, MeltWin v. 3.0. Melting temperatures (T_m) were determined by computer-fit of the first derivative of absorbance with respect to 1/T.

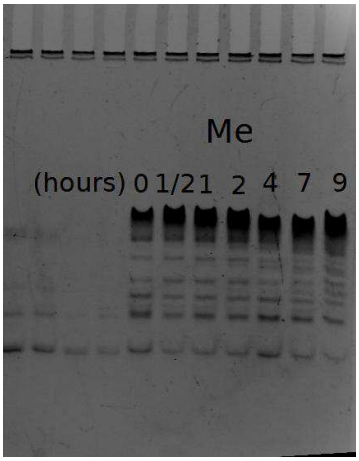
Stability of siRNAs in 50% human serum

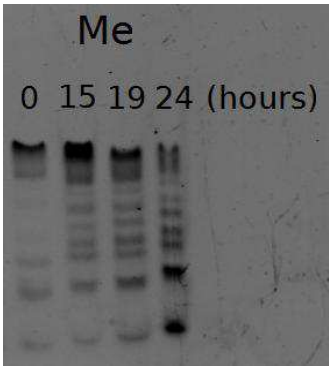
Unmodified or modified double-stranded siRNA samples (20 µM; 24 µL) were incubated in human serum (24 µL) at 37 ºC. At appropiate periods (0, 0.5, 1, 2, 4, 7 and 9 hours), 6 µL aliquots of the reaction mixture were added to 54 µL of a 1% sodium dodecyl sulphate aqueous solution and the mixtures were heated-denatured for 5 min at 90 ºC. siRNAs were isolated by hot phenol extraction followed by ethanol precipitation. In the case of F and CF3 the hot phenol extraction was skipped due to problems with the extraction. After re-suspension in 20 µL of loading buffer (90% formamide, 10% 1X TBE), the samples were run on a denaturing 14% polyacrylamide gel containing 20% formamide. RNA

bands were visualized with the SYBR Green II reagent (Sigma-Aldrich) according to the manufacturer's instructions.









RNA interference methods

SH-SY5Y and Hela cells were grown at 37 ºC, 5% CO_2 in Dulbecco's modified Eagles's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were maintained in exponential growth. The cells were plated in 24-well plates (0.5 mL medium/well) to reach about 50% confluence at transfection. The cells were grown for 24 h and the culture medium was changed to OPTIMEM 1 (GIBCO), 0.5 mL/well. Two luciferase plasmids, firefly luciferase (pGL2) and *Renilla* luciferase (pRL-CMV) from Promega, were used as reporter and control, respectively. Co-transfection of plasmids and siRNAs was carried out with Lipofectamine 2000 (Invitrogen) as described by the manufacturer for adherent cell lines. Per well, 1.0 µg pGL2, 0.1 µg pRL-CMV and the required amount of siRNAs, formulated into liposomes, were applied. The final volume was 500 µL per well. The cells were harvested 24 h after transfection, and lysed by using passive lysis buffer (PLB), 100 µL per well, according to the instructions of the Dual-Luciferase Reporter Assay System (Promega, USA). The luciferase activities of the samples were measured using a MicroLumaPlus LB 96V (Berthold Technologies) with a delay time of 2 s and an integrate time of 10 s. The volumes used were: 20 µL of sample and 30 µL of each reagent (luciferase assay reagent II and Stop & Glo Reagent). The inhibitory effects generated by siRNAs were expressed as normalized ratios between the activities of the reporter GL2 (*Photinus pylaris*) luciferase gene and the control RL (*Renilla reniformis*) luciferase gene.

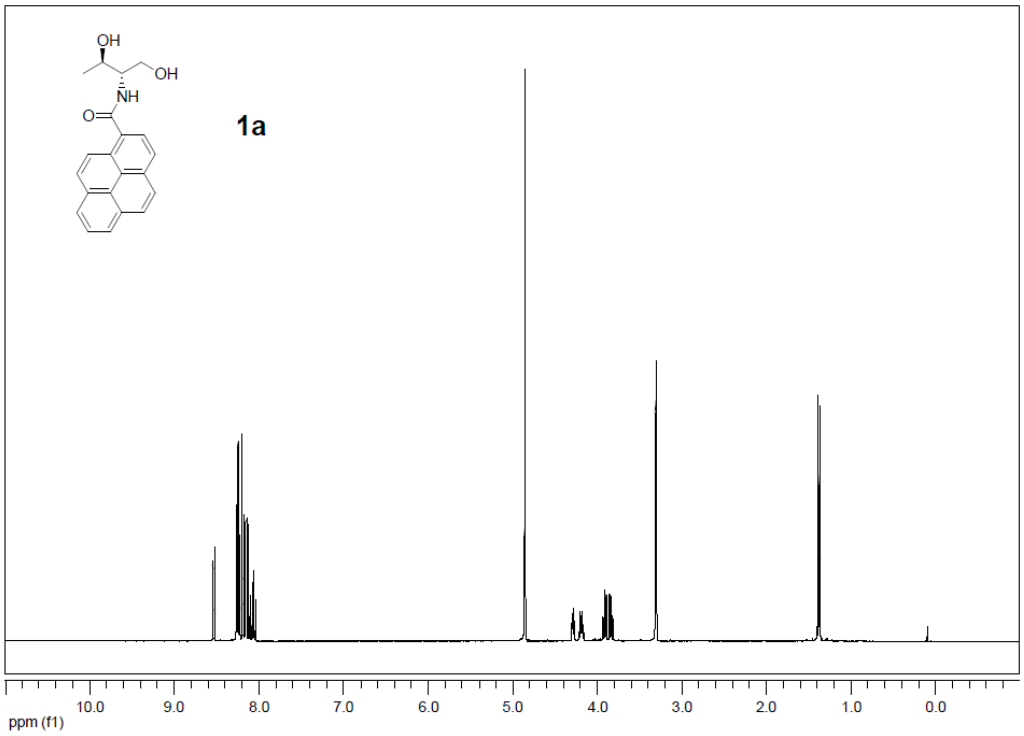
Autodock-vina docking. (O. Trott, A. J. Olson *J. Comput. Chem.* **2009**, DOI: 10.1002/jcc.21334).

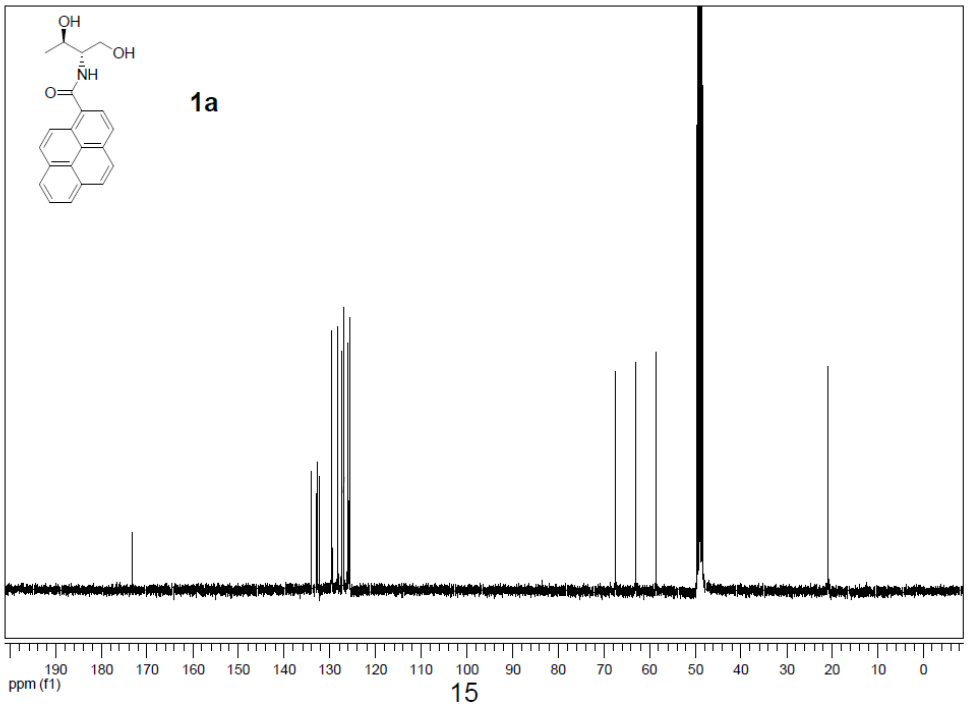
The protein (PDB: 1si3) and the ligands (pyrene, anthracene, naphthalene, trifluoromethyl benzene and fluorobenzene) were prepared for the docking using
autodock tools (ADT) (M. F. Sanner. *J. Mol Graphics Mod.* **1999**, *17*, 57-61). The following parameters were employed in the docking:

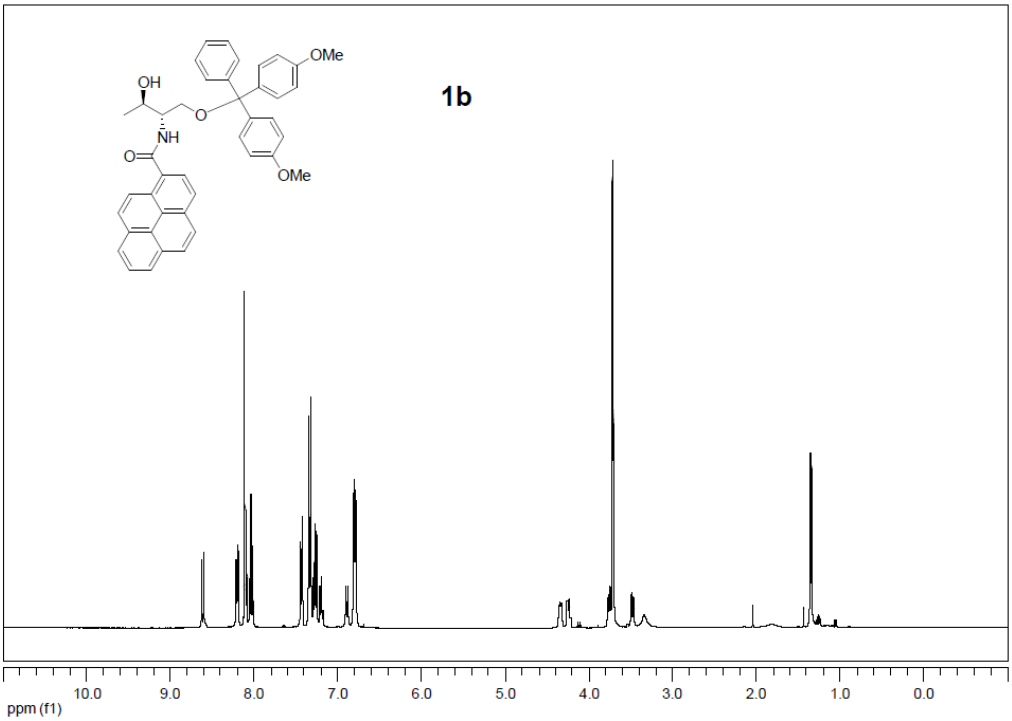
Search Space:
Center:_x = 16.837.
Center_y = 77.117.
Center_z: –13.227.
Size_x: 12 angstroms.
Size_y: 12 angstroms.

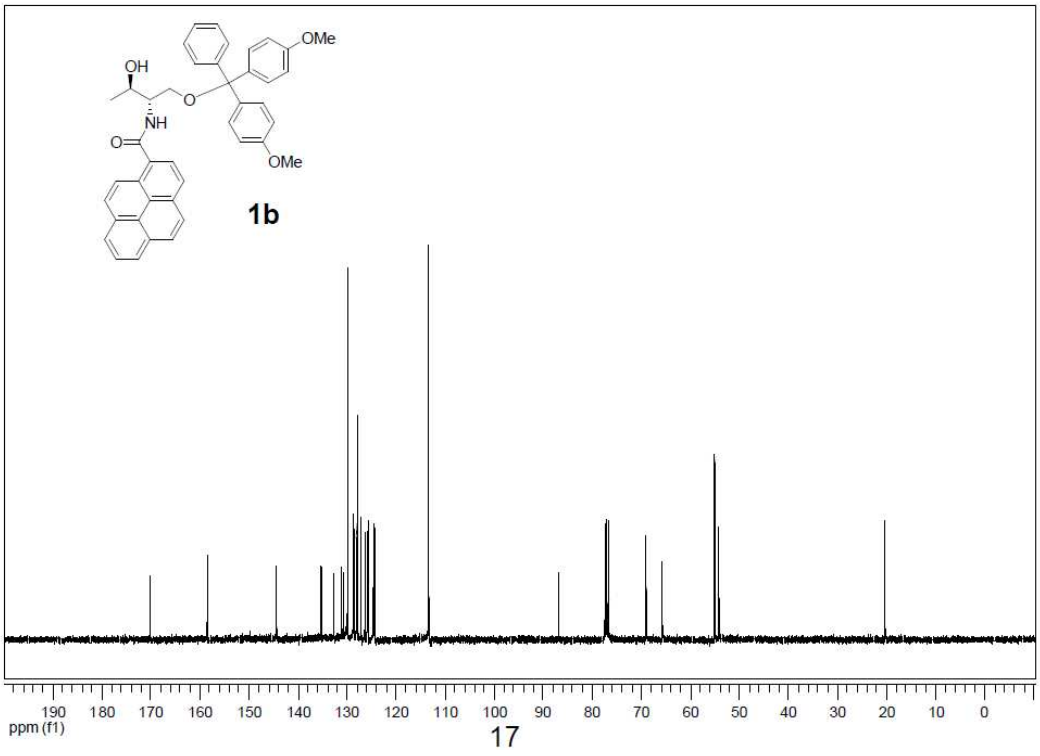
Size_z: 12 angstroms.
Exhaustiveness: 300.

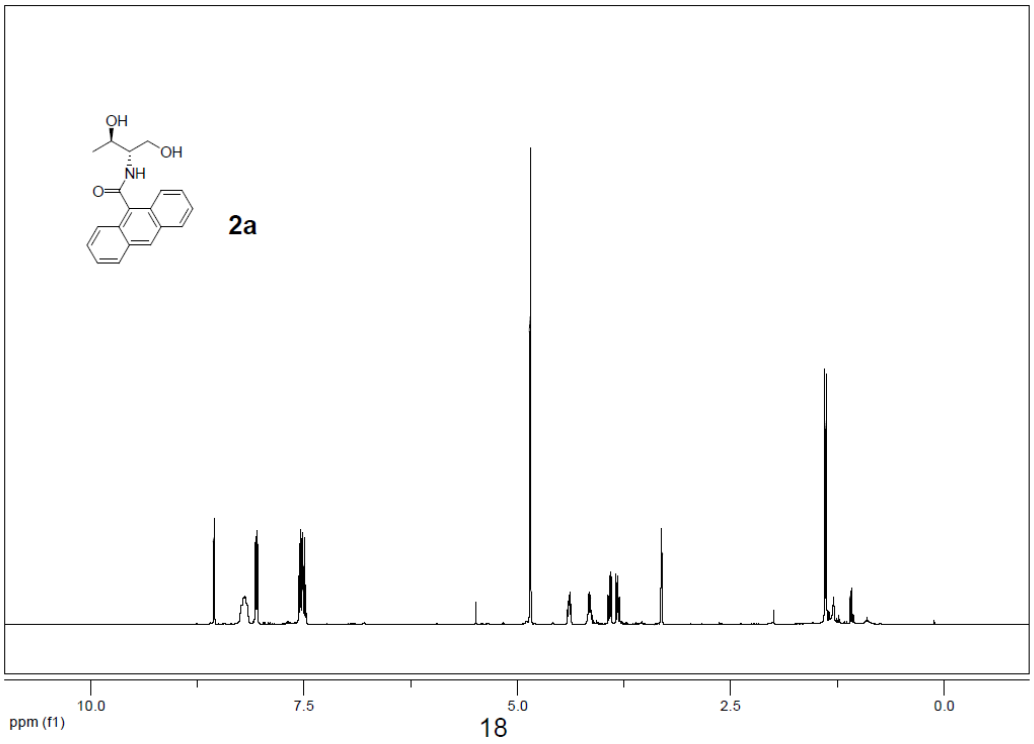
NMR SPECTRA

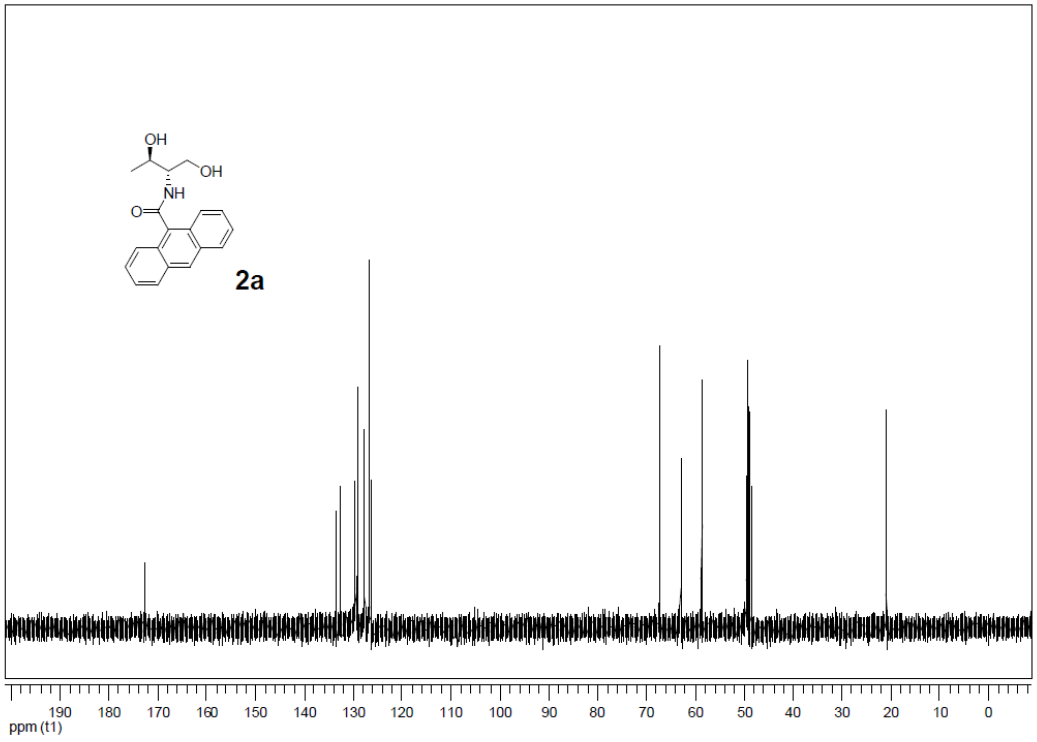


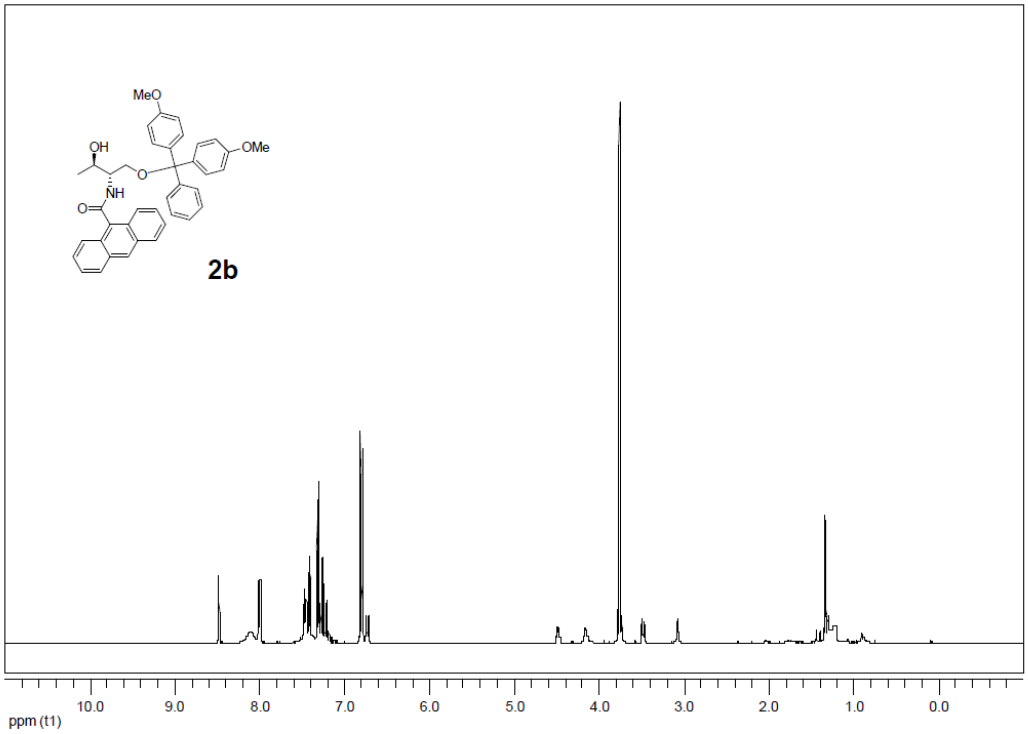

OH
OH
NH
O
1a
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm (f1)

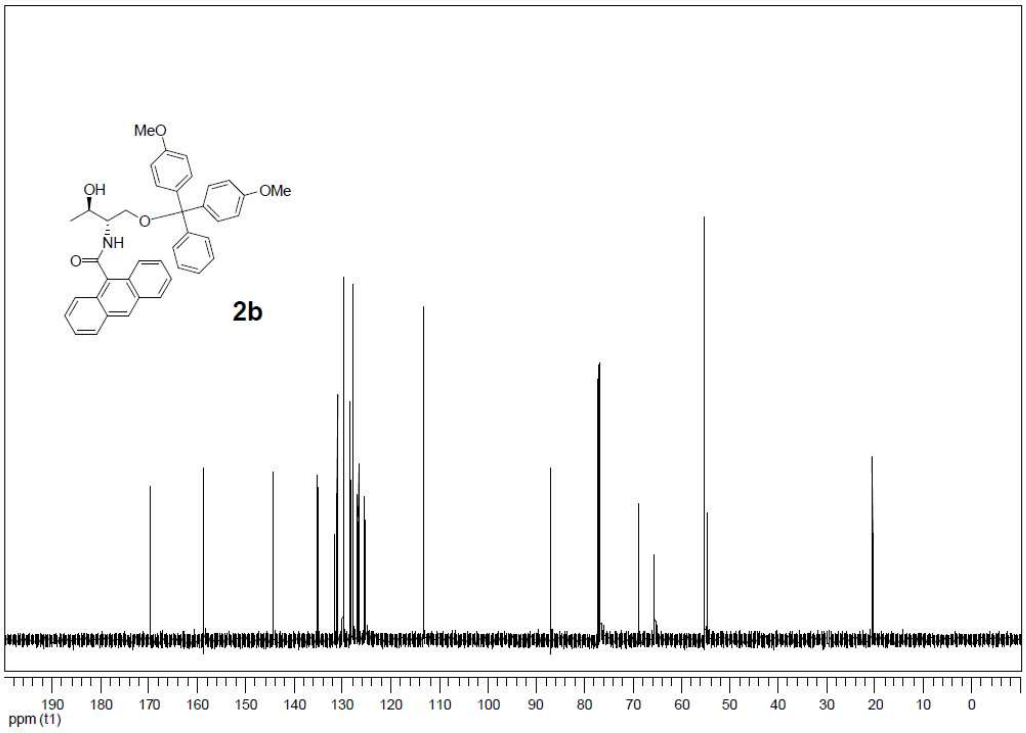

OMe
OH
O
NH
O
OMe
1b
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (f1)

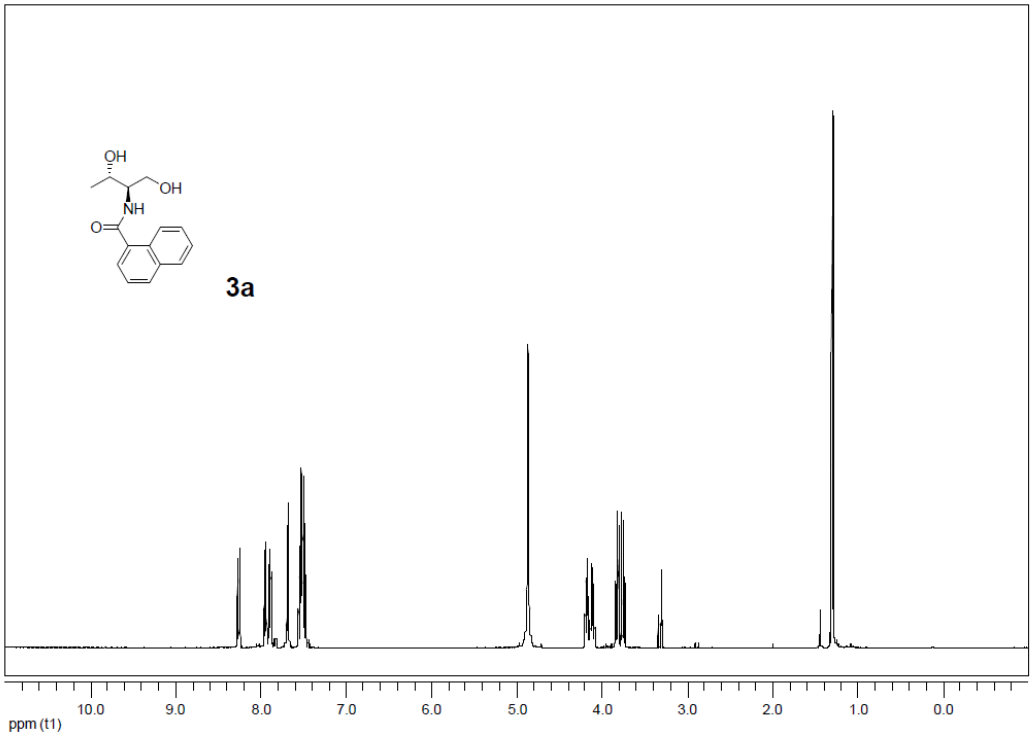

OMe
OH
O
NH
O
OMe
1b
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
ppm (f1)

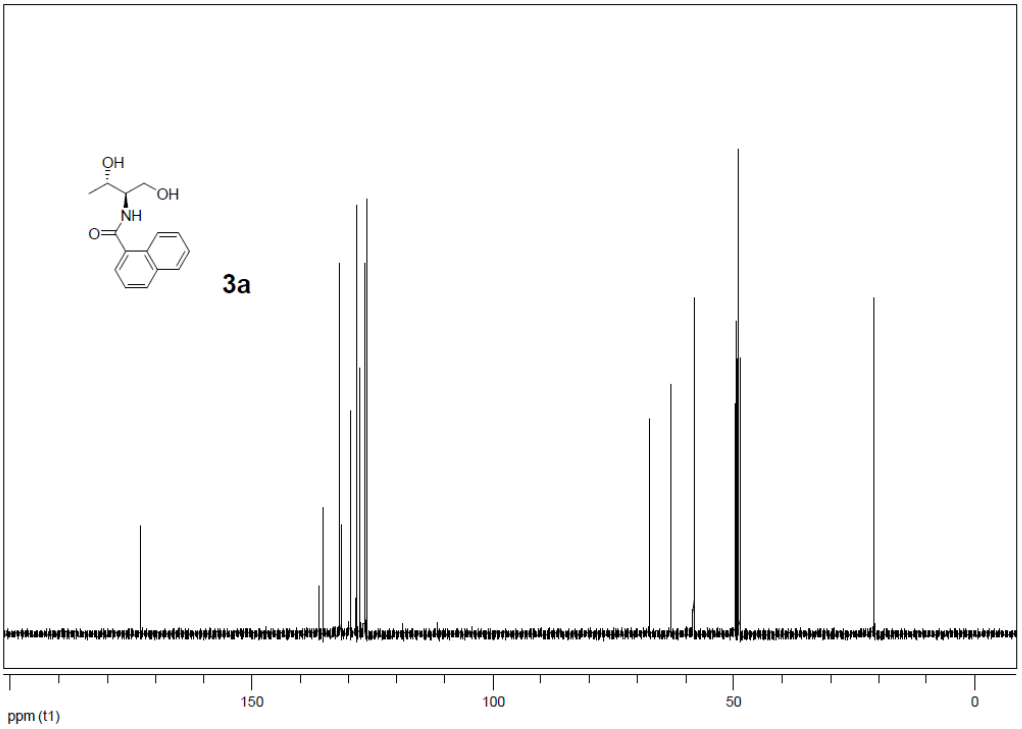

OH
OH
NH
O
2a
10.0
7.5
5.0
2.5
0.0
ppm (f1)

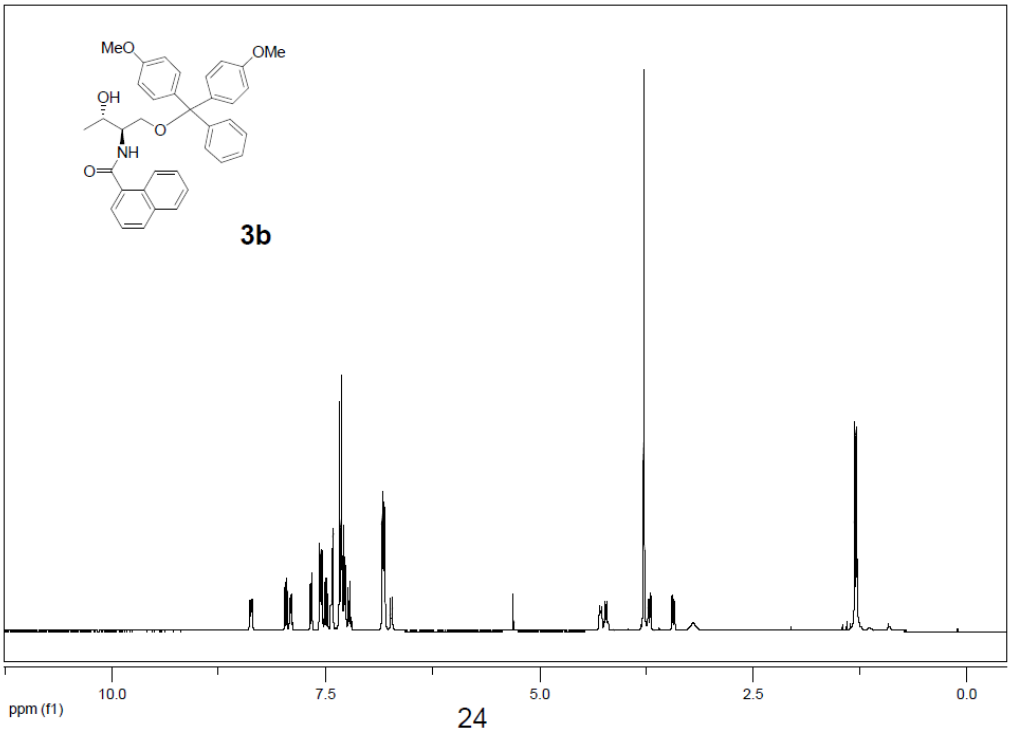

OH
OH
NH
O
2a
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm (t1)

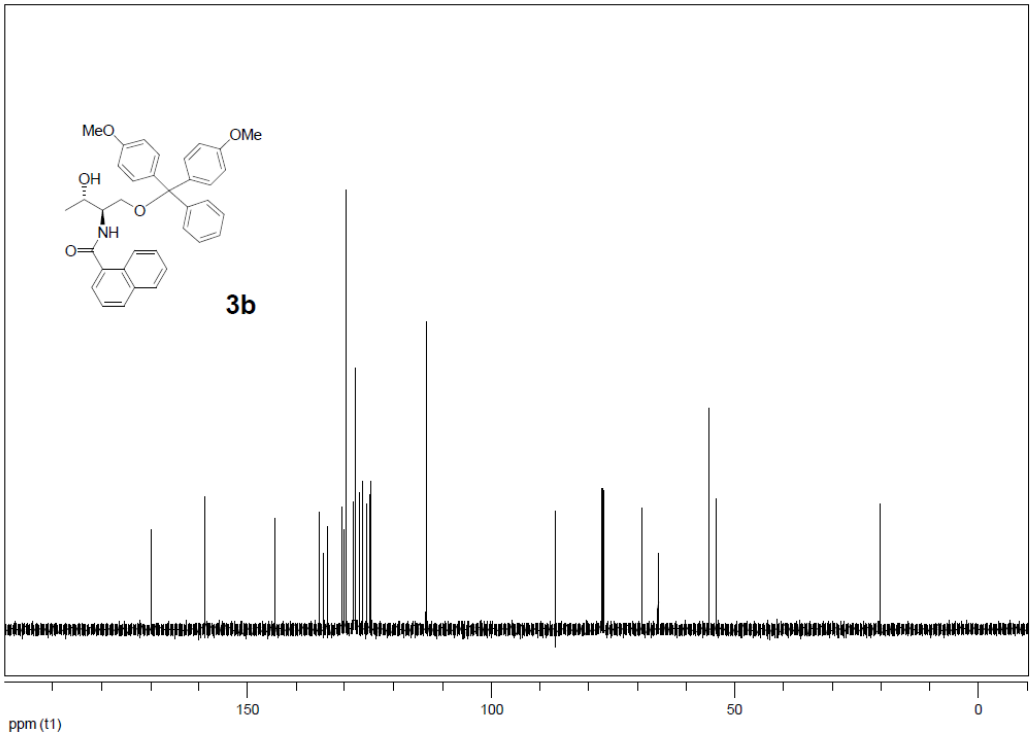

MeO
OMe
OH
O
NH
O
2b
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (t1)

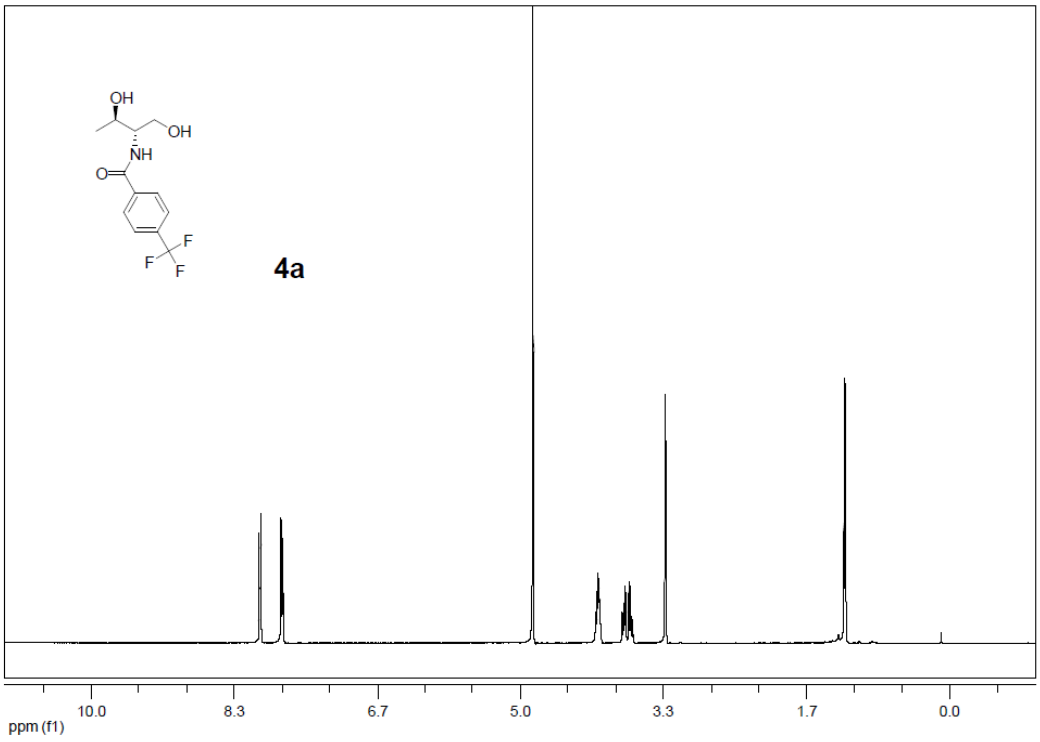

MeO
OMe
OH
O
NH
O
2b
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm (t1)

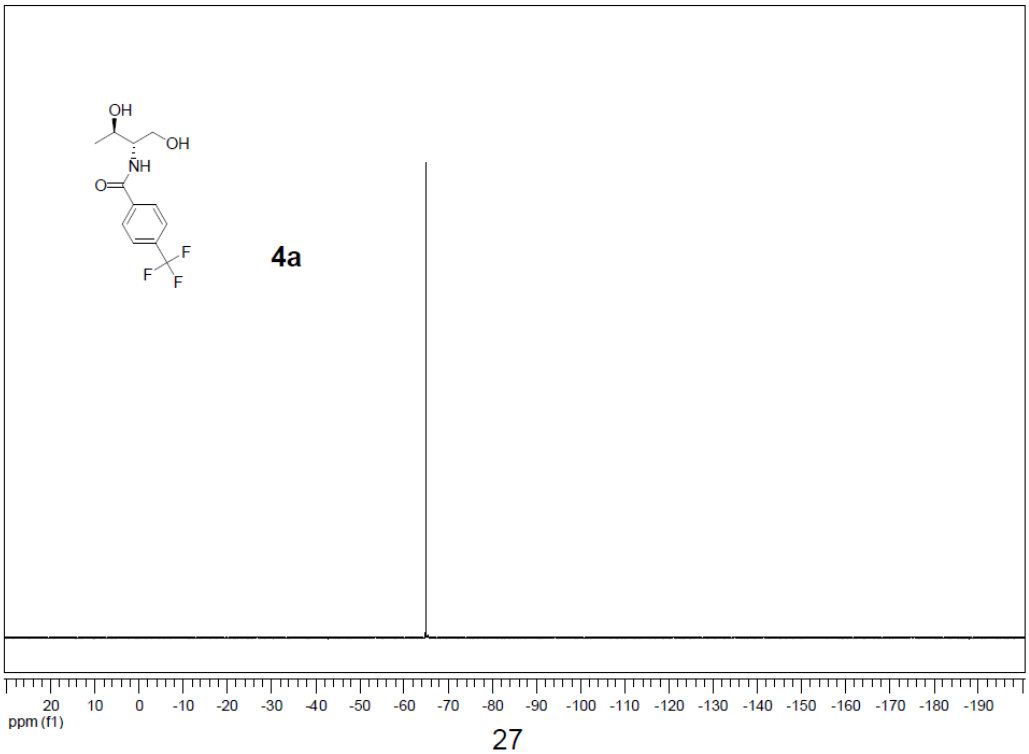

OH
OH
NH
O
3a
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (t1)

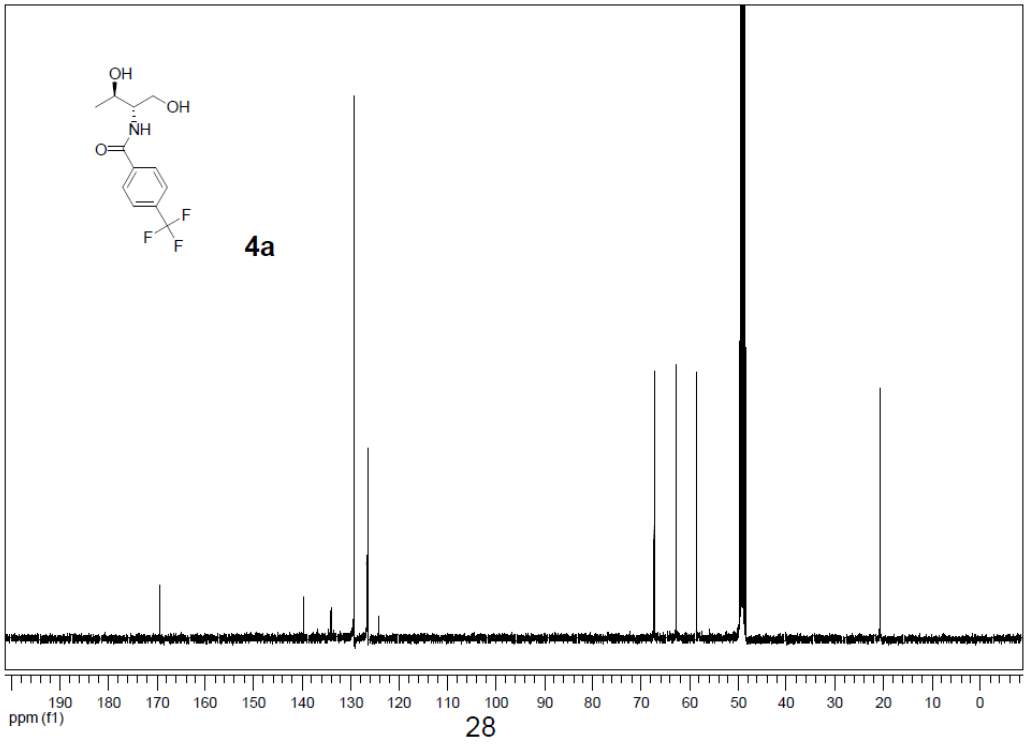

OH
OH
NH
O
3a
150
100
50
0
ppm (t1)

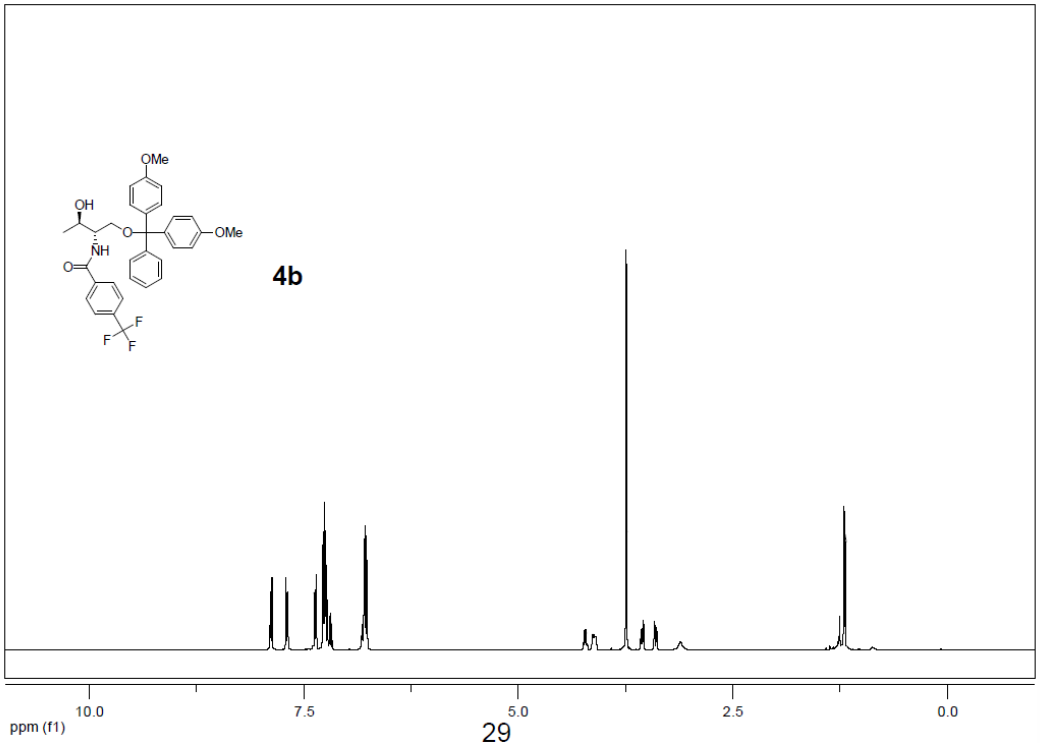

MeO
OMe
OH
O
NH
O
3b
10.0
7.5
5.0
2.5
0.0
ppm (f1)

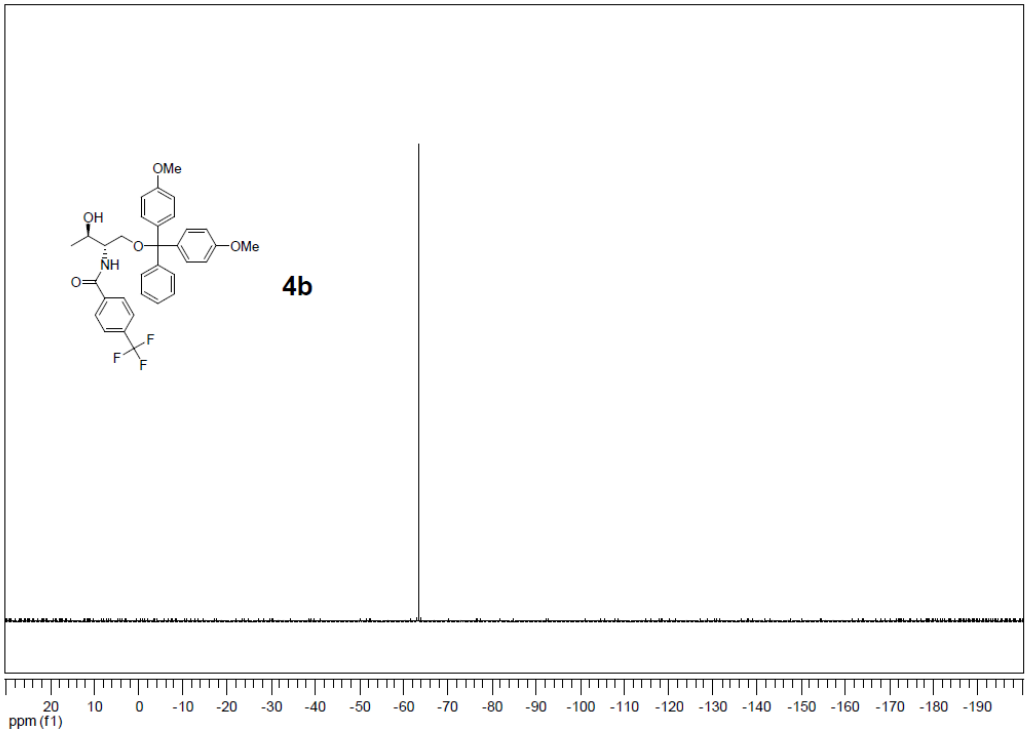

MeO
OMe
OH
O
NH
O
3b
150
100
50
0
ppm (t1)

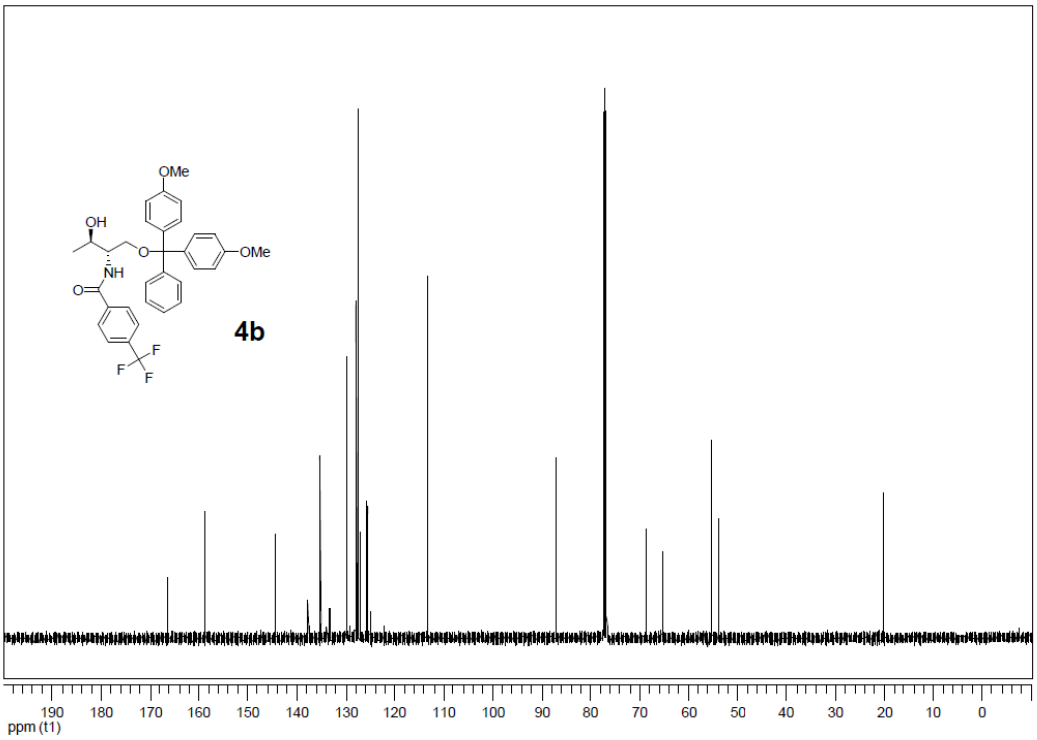

OH
OH
NH
O
F
F
F
4a
10.0
8.3
6.7
5.0
3.3
1.7
0.0
ppm (f1)

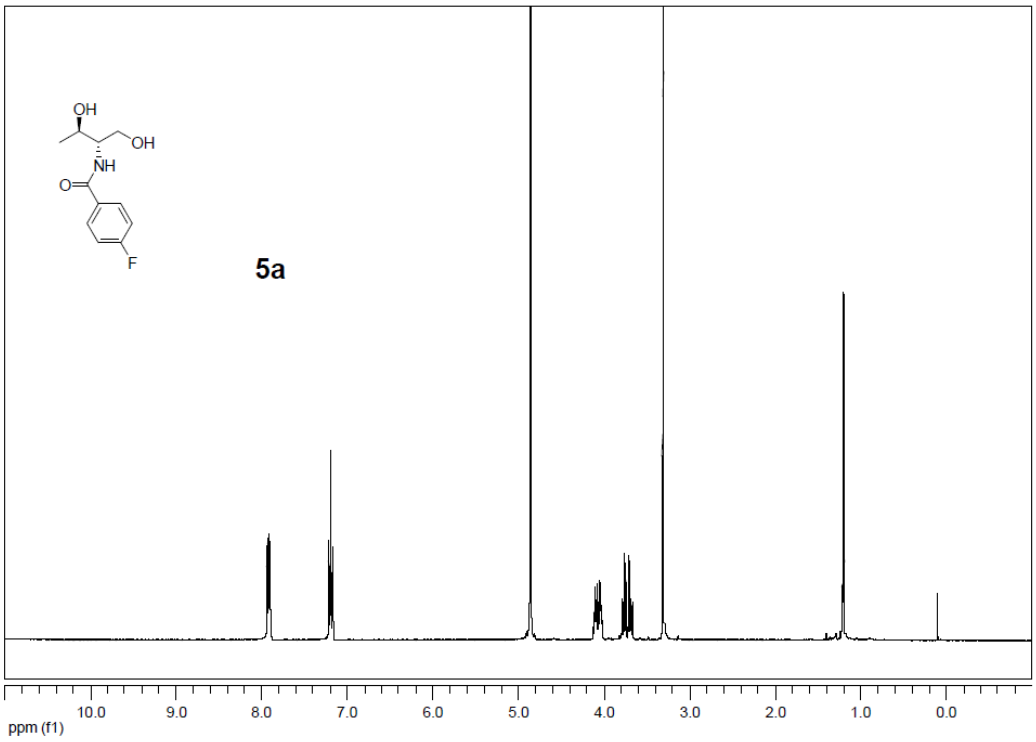

OH
OH
NH
O
F
F
F
4a
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
-150
-160
-170
-180
-190
ppm (f1)

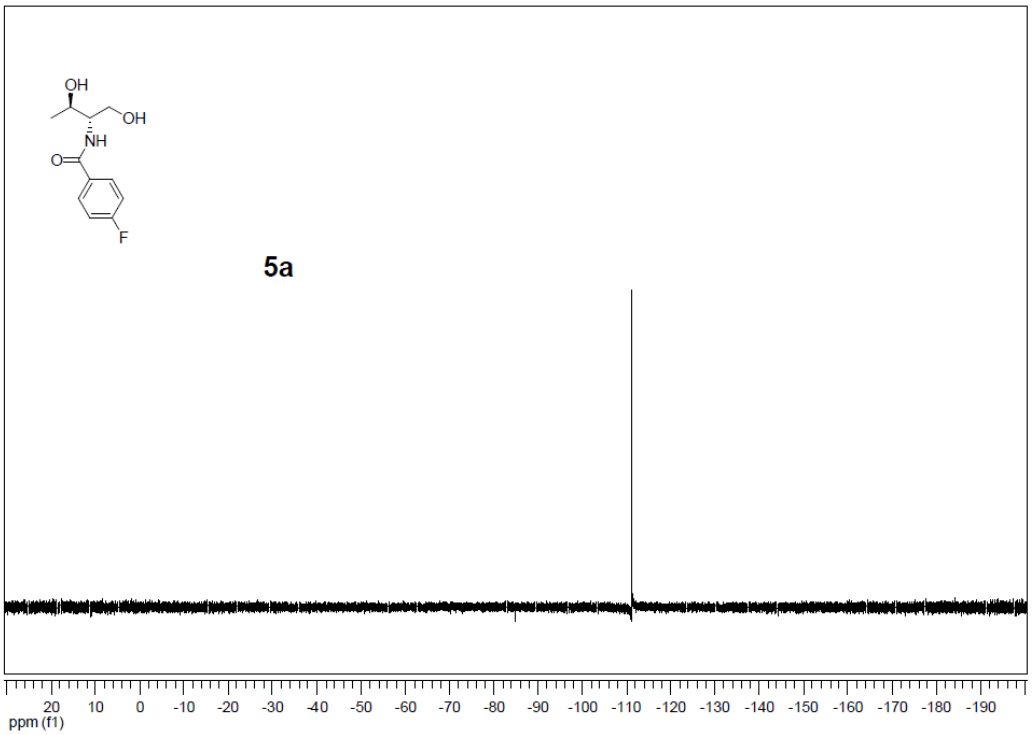

OH
OH
NH
O
F
F
F
4a
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
ppm (f1)

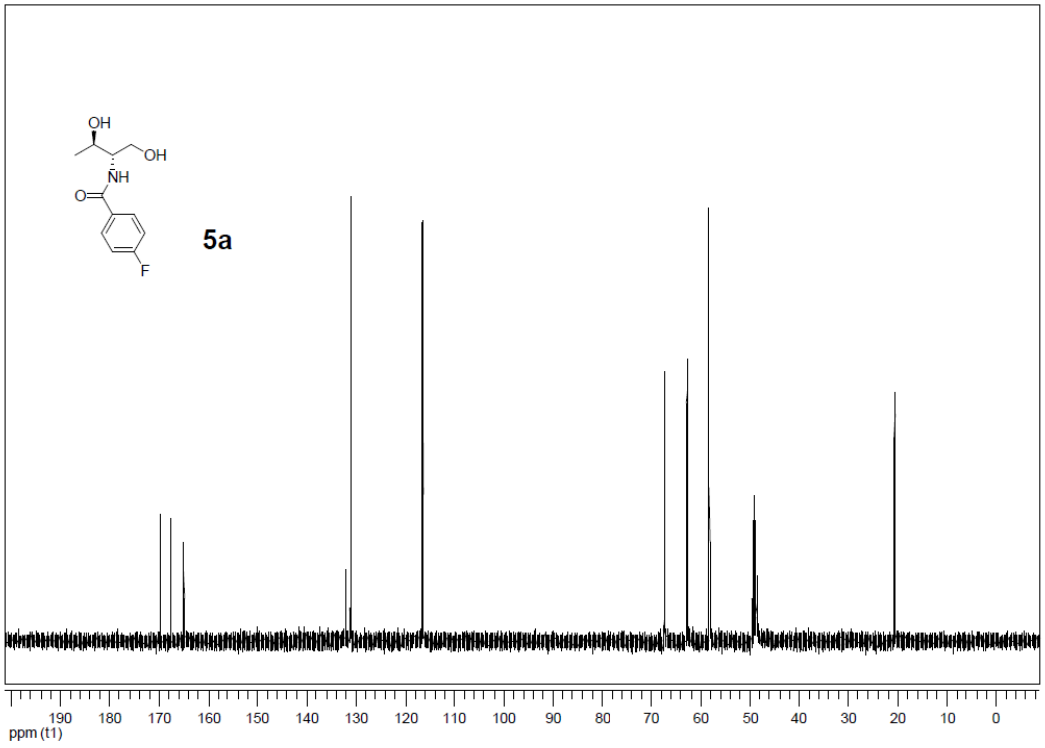

OMe
OH
O
OMe
NH
O
F
F
F
4b
10.0
7.5
5.0
2.5
0.0
ppm (f1)

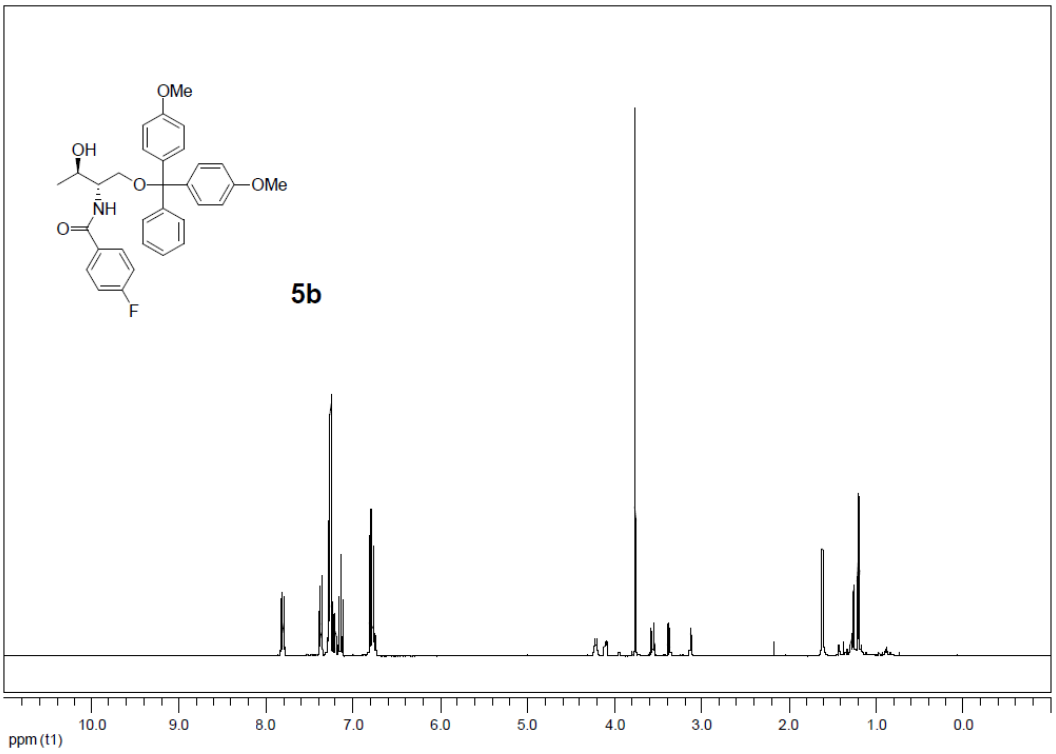

OMe
OH
O
OMe
NH
O
F
F
F
4b
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
-150
-160
-170
-180
-190
ppm (f1)

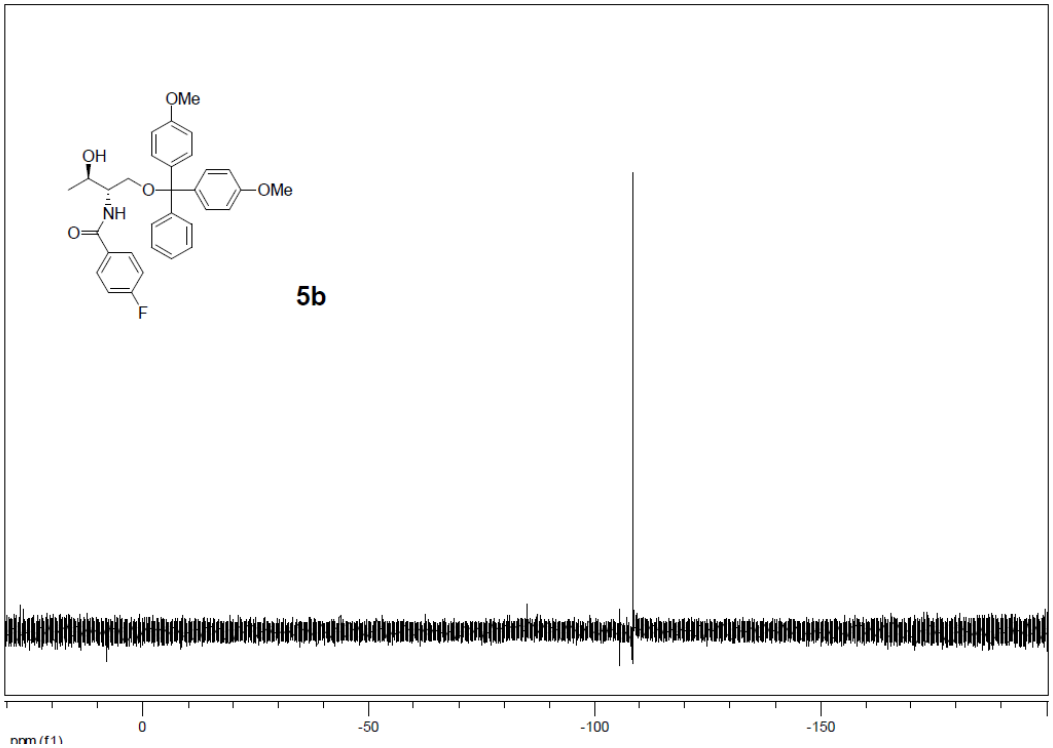

OMe
OH
O
OMe
NH
O
F
F
F
4b
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm (t1)

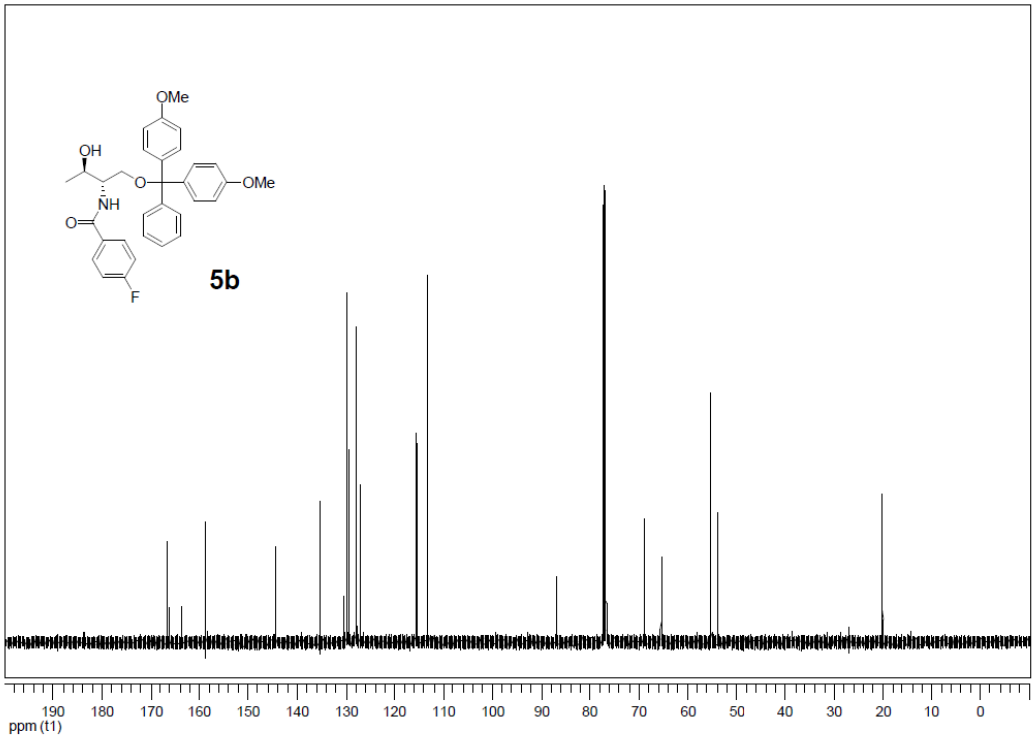

OH
OH
NH
O
F
5a
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (f1)

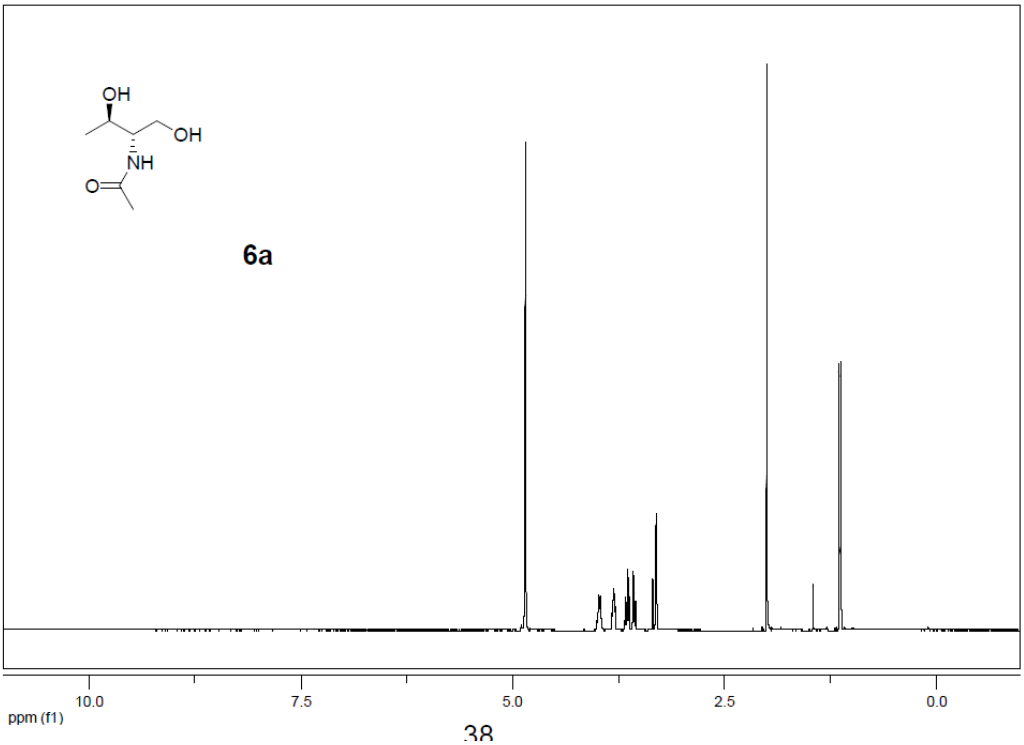

OH
OH
NH
O
F
5a
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
-150
-160
-170
-180
-190
ppm (f1)

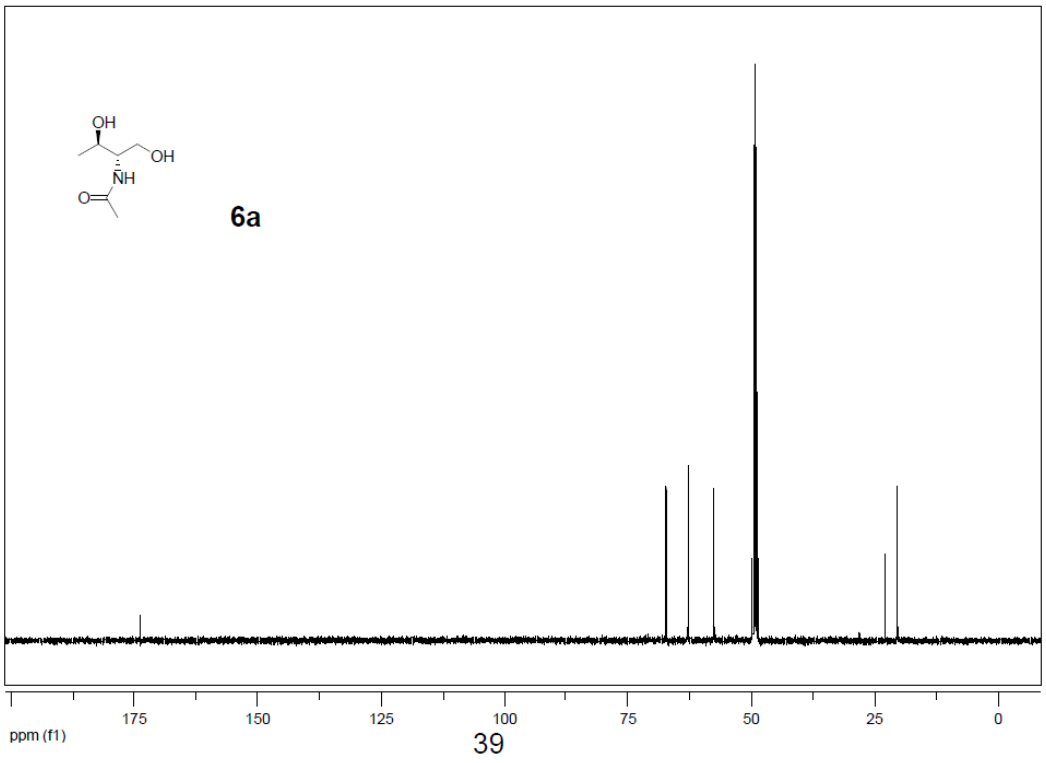

OH
OH
NH
O
F
5a
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
ppm (t1)

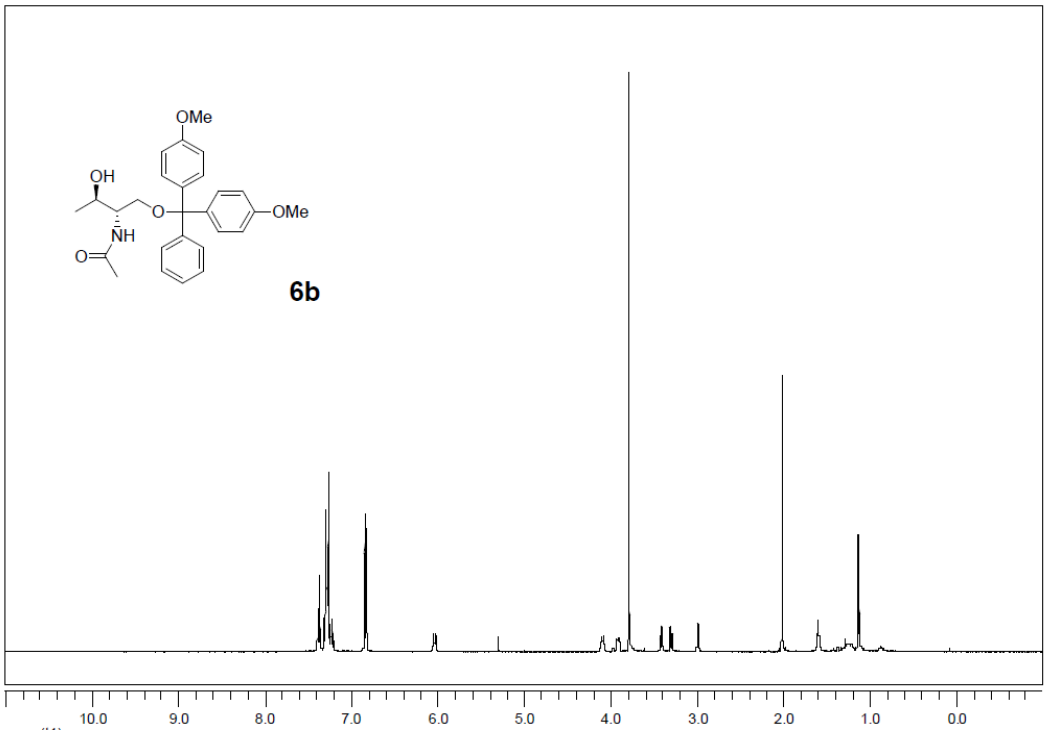

OMe
OH
O
OMe
NH
O
F
5b
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (t1)

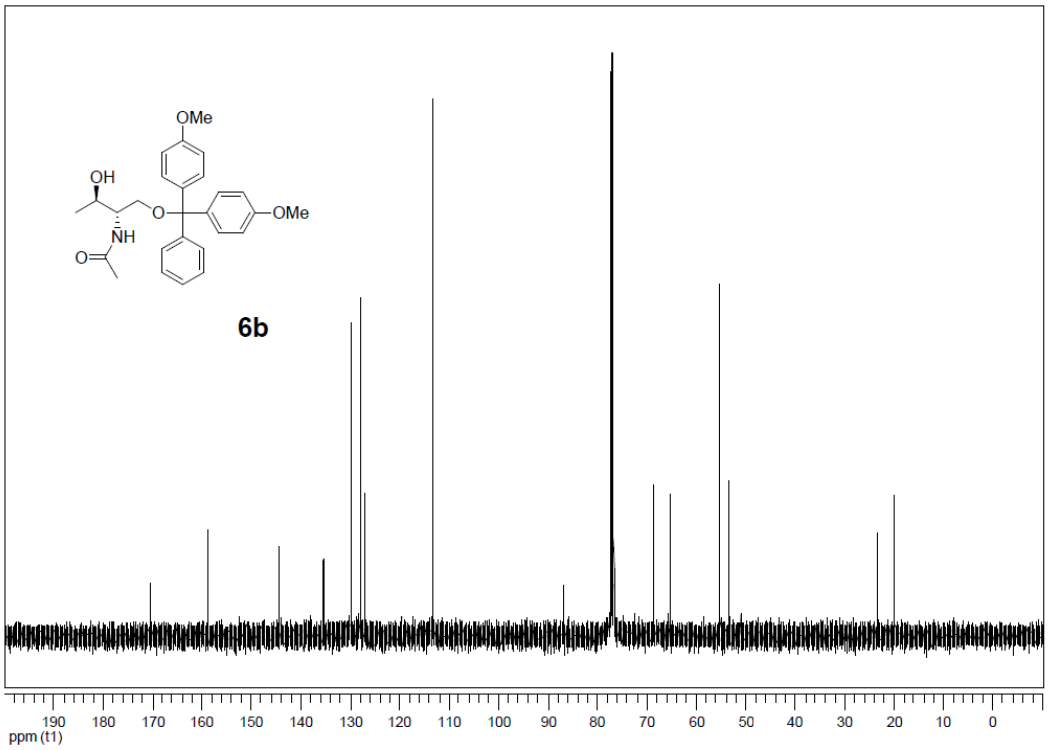

OMe
OH
O
OMe
NH
O
F
5b
0
-50
-100
-150
ppm (f1)


OMe
OH
O
OMe
NH
O
F
5b
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm (t1)


OH
OH
NH
O
6a
10.0
7.5
5.0
2.5
0.0
ppm (f1)


OH
OH
NH
O
6a
175
150
125
100
75
50
25
0
ppm (f1)


OMe
OH
O
OMe
NH
O
6b
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0


OMe
OH
O
OMe
NH
O
6b
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm (t1)